COMMISSION ON IMMUNIZATION

ANNUAL REPORT TO THE ARMED FORCES EPIDEMIOLOGICAL BOARD

1970 - 1971

Additional copies of this bound volume are not available. However, copies of the investigators' reports, herein, may be requested in accordance with the DDC distribution statement on the individual front covers.

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ARMED FORCES EPIDEMIOLOGICAL BOARD

COMMISSION ON IMMUNIZATION

DIRECTOR'S SUMMARY REPORT - FALL 1971

On March 26, 1971, an executive session of the Commission on Immunization was held at Walter Reed Army Institute of Research, reviewing the Commission missions and contract applications. Dr. Willard F. Verwey was recommended for appointment as a full member; this recommendation has been approved by the Board. Dr. K. F. Meyer was recommended as advisory member. Drs. Arthur M. Silverstein, Bernard B. Levine and H. Bruce Dull were recommended as associate members.

The annual technical meeting was held as a joint meeting with the Commission on Epidemiological Survey on 23 and 24 September 1971, at the Walter Reed Army Institute of Research. A program reviewing the Commission sponsored research, the status of the areas of interest to our Commission and the immunological studies being carried out under the purview of the Commission on Epidemiological Survey.

I. Annual Meeting

a. Agenda (Commission on Immunization Presentations - Presenters are underlined)

September 23, 1971

Introductory Remarks - Commission Directors, President and Executive Secretary of the Board

Reports of Preventive Medicine Officers
    Department of the Army
    Department of the Navy
    Department of the Air Force

Report, Representative of USA Med. R & D Command

The VEE Story	Colonel Dan Crozier, MC, USAMRIID Lt. Colonel Richard O. Spertzel, VC USAMRIID

Scientific Presentations - Antibodies

Antibody Formation and Immunity	Dr. Jonathan W. Uhr N.Y.University School of Medicine
Analysis of Sequential Humoral Antibody Responses	Mrs. Mary H. Wilke, USAMRIID
Local Respiratory and Humoral Responses to Immunization	Major Stanley H. Rabinowitz, MC USAMRIID
Electron Spin Resonance Studies of Antigen-Cell Membrane Interaction	Major Joseph Kaplan, MC, USAMRIID Captain William J. Caspary, MSC USAMRIID
Classes and Properties of Human Antibodies	Dr. Bernard B. Levine N.Y. University School of Medicine
Use of Microprecipitation and Electrophoresis in Diagnostic Virology	Captain Neil H. Levitt, MSC, USAMRIID
Development of the Immune Response	Dr. Arthur M. Silverstein The Johns Hopkins University School of Medicine

Studies of Cell Mediated Immunity

Mitogenic Effects of Enterotoxins in Lymphocytes	Major William H. Adler, III, MC USAMRIID
Mechanisms of Delayed-type Hypersensitivity	Dr. Alvin Volkman Trudeau Institute, Inc.
An Oral Mouse-Protection Test for the Assessment of Typhoid Vaccines	Dr. Frank M. Collins Trudeau Institute, Inc.

September 24, 1971

Studies of Cell Mediated Immunity (continued)

Physicochemical Studies of the Biological Significance of Antiviral Antibody	Dr. Joseph A. Bellanti Georgetown University School of Med.

Studies of Cell Mediated Immunity (continued)

Lysosomal Responses during Infection	Captain Peter G. Canonico, MSC USAMRIID
Cellular and Humoral Immunity in Parasitic Infections	Dr. Noel R. Rose State University of N.Y. at Buffalo
Hypercholesterolemia and Altered Immunity	Dr. William R. Beisel, USAMRIID
Endogenous Mediators of Nonfebrile Host Responses	Dr. Robert S. Pekarek, USAMRIID

Scientific Presentations - Vaccine Development

Factors Influencing the Pattern of the Immune Response	Dr. Geoffrey Edsall Harvard School of Public Health
Spotted Fever Vaccine	Dr. Richard H. Kenyon, USAMRIID
Live Q Fever Vaccine	Major David M. Robinson, VC USAMRIID

Scientific Presentations

Hemorrhagic Fever in Cochabamba	Dr. C. J. Peters Middle America Research Unit NIAID
Staphylococcal Enterotoxoids	Colonel Joseph F. Metzger, MC USAMRIID
Pathogenesis and Control of Enteric and Rickettsial Infections in Volunteers	Dr. Richard B. Hornick University of Maryland School of Med.
Isolation and Characterization of Lymphogranuloma Venereum Agents	Dr. Karl F. Meyer(presented by Dr. Julius Schachter)* University of California

Scientific Presentations (continued)

Mechanisms of Inflammatory Response in Man	Dr. Sheldon E. Greisman University of Maryland School of Medicine

Other Items of Interest:

Gammopathy
Smallpox
B Virus
Rabies
Mumps

Executive Sessions

* Dr. Karl F. Meyer was unable to attend; Dr. Julius Schachter reported for him.

Dr. Herman N. Eisen (Cell Mediated Immune Responses) and Dr. Nicholas P. DiLuzio (Role of the RES and Humoral Recognition Factors in Malaria) were unable to attend the meeting; their reports are included.

b. Attendance

Members Commission on Immunization	September 23	24
K. Frank Austin	A	P
Elmer L. Becker	A	A
Abram S. Benenson	P	P
*Geoffrey Edsall	P	P
Herman N. Eisen	A	A
Thomas J. Gill, III	P	P
C. Henry Kempe	P	P
*Irwin H. Lepow	A	A
George B. Mackaness	P	P
*Karl F. Meyer	A	A
Robert O. Oseasohn	P	P
Jonathan W. Uhr	P	P
Willard F. Verwey	P	P

* Advisory Members

Associate Members Commission on Immunization		
Albert H. Coons	A	A
H. Bruce Dull	P	A
William S. Gochenour, Jr.	P	P
Samuel L. Katz	A	A
Bernard B. Levine	P	A
John D. Marshall, Jr.	P	P
Abraham G. Osler	A	A
Alwin M. Pappenheimer, Jr.	P	P
Margaret Pittman	A	A
Elvio H. Sadun	P	A
Arthur M. Silverstein	P	A
Jay P. Sanford	P	P
John C. Wagner	P	A
Fred S. Rosen	P	A

Responsible Investigators Commission on Immunization		
William A. Altemeier, III	A	A
Joseph A. Bellanti	A	P
Frank M. Collins	P	P
Nicholas R. DiLuzio	A	A
Noel R. Rose	P	P

Army Representatives		
CPT Thomas H. Hudson, USAMRIID	P	P
CPT Neil H. Levitt, USAMRIID	P	P

Army Representatives (continued)

	September 23	24
MAJ Carl E. Pedeisen, Jr., USAMRIID	P	A
MAJ David Robinson, USAMRIID	P	A
MAJ Joseph Kaplan, USAMRIID	P	A
LTC Harry G. Dangerfield, USAMRIID	P	P
LTC Richard Spertzel, USAMRIID	P	P
MAJ Stanley G. Rabinowitz	P	A
COL Dorsey T. Mahin, USAMRDC	P	P
COL Richard F. Barquist, USAMRDC	P	A
COL Donald W. Sample, USAMRDC	P	P
MAJ William H. Adler, USAMRIID	P	P
MAJ Charles T. Kaelber, OTSG/DA	P	P
COL Robert L. Krivulka	P	P
LTC Peter Bartelloni, USAMRIID	P	A
MAJ John R. Warren, USAMRIID	P	A
CPT O. J. Paisled, WRAIR	A	P
COL J.F. Metzger, USAMRIID	A	P
CPT P. G. Canonico, USAMRIID	A	P

Navy Representatives

LT J. C. Hill, NMRI	P	A
CAPT Charles E. Alexander, BuMed	P	P
LCDR John Poundstone, BuMed	P	A

Air Force Representatives

COL Paul F. Nugent, OTSG/USAF	P	A
LT COL Frank T. Corker, USAF/SGPAAP	P	A

Armed Forces Epidemiology Board

Gustave J. Dammin, President	P	P
Bradley W. Prior, Col, USAF, Exec Secty.	P	P
Floyd W. Denny, Board Representative	P	P

Guests

LTC John K. Read, AFIP	P	A
COL Hinton Baker, WRAIR	P	A
Francis E. Cole, Jr., USAMRIID	P	A
COL H. W. Whitcher, Br Med Lia	P	P
Richard H. Kenyon, USAMRIID	P	P
Virginia G. McGann, USAMRIID	P	P

Guests (continued)	September 23	24
Leonard Spero, USAMRIID	P	P
Robert Wannemacher, USAMRIID	P	P
Robert S. Pekarek, USAMRIID	P	P
Richard H. Zeschke, SUNY/Buffalo	P	P
Howard Noyes, WRAIR	P	P
COL Philip K. Russell, WRAIR	P	P
Walter E. Brandt, WRAIR	P	A
MAJ Robert Scott, WRAIR	P	A
Arthur J. Emery, Jr., Ofc Naval Res	P	A
John Schachter, Univ Cal Med Sch	P	P
Michael B. Gregg, CDC	P	A
Harold N. Glassman, Bio Def Res Lab	P	P
Carl Lamanna, OCRD/DA	P	A
Daniel H. Connor, AFIP	P	A
Kathleen Kos, WRAIR	P	A
MAJ Daniel Shapiro, WRAIR	P	A
S. B. Formal, WRAIR	A	P
M. H. Wilkie, USAMRIID	P	P
William R. Beisel, USAMRIID	A	P
COL T. H. Lamson, WRAIR	A	P

II. Commission Sponsored Research

The annual progress reports of Commission-sponsored contracts and the final report of Dr. Meyer's contract on "Plague" are appended to this report. Highlights of the individual reports and the discussions at the meeting are presented here.

a. Dr. J. W. Uhr (Antibody Formation and Immunity) reported on the studies that have been carried out in his laboratories. Studies on splenic lymphocytes led him to conclude that these cells form an immunoglobulin which is present on the cell surface and gives the lymphocyte the capacity to bind antigens specifically. Over 95% of the immunoglobulin from the small lymphocyte fraction is IgM; this IgM exists on the surface of the small lymphocyte as a 7-8S monomer. Studies with antitheta serum and "pure" B and T cells show that these IgM molecules come from the surface of B lymphocytes primarily - these cells are the precursors of antibody-secreting cells and the antibody which will be secreted is predominantly IgG.

Immunoglobulin synthesis was studied in a Burkitt lymphoma cell. The immunoglobulin is synthesized by polyribosomes bound to the Golgi complex, where galactose and fucose are added and is attached to membranes throughout its intracellular existence. It reaches the cell surface where the Fc portion attaches to the plasma membrane and the Fab portion is exteriorized and available for antigen binding. This sequence is essentially the same as that with a secreting cell except that here the immunoglobulin is not released from the plasma membrane.

His studies on regulation of antibody formation suggested to Dr. Uhr a model in which free antigen, free antibody and antigen-antibody complexes exist in various ratios; immunogenicity is inversely related to the antibody-antigen ratio. This would explain the overshoot phenonema he observes when he depletes an antibody by exchange transfusion, and the tendency for antibody levels to oscillate over time rather than hold a constant level.

b. Dr. B. B. Levine (Classes and Properties of Human Antibodies) has continued his work in mice defining the factors which control the production of reagins (IgE). The IgE response to a small dose of antigen which is observed with some mouse strains is controlled by a single autosomal locus closely linked to the H-2 type. This response is antigen-specific and occurs after low doses of the antigen to which that mouse strain will respond. With a large dose of antigen, some strains produce primarily IgG, while other mouse strains produce both IgG and reagin; this response, which is not antigen-specific, appears from cross breeding experiments (in which the backcross results in good producers, poor producers and intermediate producers) to be a multilocus effect. Administered intraperitoneally with alumina gel in the proper mouse line, 0.1 microgram of ovalbumin with one booster elicited reagin persisting for at least six months. The ratio of IgG to reagin is increased by giving the antigen weekly rather than every four weeks. Haptenic antigen injected intraperitoneally with alumina gel in repeated small doses elicited homocytotropic antibodies of the IgE type in guinea pigs and in rabbits. This work is providing excellent models for the study of reagin and atopic allergy in man.

c. Dr. A. M. Silverstein (Development of the Immune Response) has continued his studies defining the ontogeny of the immune process. He had previously shown that the fetal lamb can begin to produce IgM and IgG antibodies against phage as early as 41 days of gestation while other antigens, such as diphtheria toxoid, *Salmonella typhi* antigen or BCG, do not constitute adequate antigenic stimuli until several weeks after birth. Preliminary studies suggest that IgA antibody can be formed in the fetal tissue in response to appropriate antigenic stimulus within the intestine. A technique is under investigation which involves the implantation of a small portion of ileum into the anterior chamber of the eye where demonstration of IgA would prove that local differentiation of IgA forming cells from lamina propria of the implanted gut has occurred, since only IgM and IgG occur in ocular tissue. Morphological studies have indicated that germinal center formation is not essential for primary antibody response or for the establishment of persisting immunologic memory. Germinal centers in Peyer's patches result from stimulation from the lumen, since a mid-ileal atresia suppressed the formation of Peyer's patches in the distal ileum and antigenic stimulation by feeding the fetus through an exteriorized catheter into the intestinal lumen resulted in precocious maturation of germinal centers.

Inoculation of the early fetal lamb with lymphocytic choriomeningitis virus produces cytopathogenic encephalopathy. As the fetus matures and active antibody formation occurs, there is clearance of virus from the circulation. Thymectomy followed by administration of antilymphocytic serum prevents the formation of antibodies and a fetal viremia occurs, which persists into the neonatal period; but CNS pathology does not occur despite high and persisting virus titer in the brain. Bluetongue virus produces encephalopathy when introduced in the early gestation period but later is innocuous. It now appears that the adverse effects occur during migration of cells from the ependymal plate. This cell migration and developmental susceptibility terminate at about the same time that the fetus begins to form circulating antibody against the viral antigens.

d. Dr. F. M. Collins (An Oral Mouse Protection Test for the Assessment of Typhoid Vaccines) has been using the Salmonella enteritidis-mouse system as a model for the study of typhoid fever in man. When susceptible mouse strains are given an oral challenge with a relatively small number of virulent organisms, there is a delay in spread from the gut to the liver and spleen, and when these organisms increase in these organs, death follows. On the other hand an avirulent organisms such as Salmonella gallinarum given intravenously tend to survive in these organs for a period but then decline to zero; but even when given orally small numbers can be recovered from the liver and spleen. The variation in susceptibility of various mouse strains would seem to relate to the facility with which the organisms can pass through the gut wall and then the ability of the animal to control those which do reach the liver and the spleen. In studies designed to follow the movement of organisms closely, it was interesting that after the gastric acidity was neutralized by bicarbonate, organisms could be demonstrated within the stomach walls after they were no longer recoverable from the stomach contents. When the mice were vaccinated with a killed vaccine, there was little effect, but perhaps some reduction in the rate in which the organisms leave the gut despite good O-agglutinating titers. When a strain of Salmonella enteritidis of lower virulence but yet which still penetrated through the gut and persisted in the liver, spleen and lymph node was used in animals immunized with heat-killed organisms, the gut penetration was definitely inhibited and the rate at which the organism multiplied in the liver and spleen was slowed. Earlier infection with the attenuated Salmonella enteritidis strain afforded protection against oral challenge with the virulent strain only if the original dose was enough to give a good systemic reaction. When the challenge was too low, the animals remained completely susceptible to reinfection. The part played by secretory immunoglobulins in this protection has not been completely defined. A question has been raised in view of the usually rapid transit time through the stomach in the mouse whether bicarbonate had an activity other than that of protecting the challenge organisms from gastric acidity.

e. Dr. J.A. Bellanti (Physicochemical Studies and Biologic Significance of Antiviral Antibody) has been conducting studies on viral infections for a period of time but because these have become more and more concerned with the immunological phenomena involved, sponsorship this year came to the Commission on Immunization. In his report he discussed the phenomena involved in phagocytosis, particularly as typified by chronic granulomatous disease in which phagocytosing leukocytes have a biochemical lesion which involves the hexose-monophosphate shunt preventing eventual production of hydrogen peroxide. The defect can be recognized by the lack of the oxidation of nitro-blue tetrazolium to formazan pigment which occurs in the normal leukocyte. Using sand fly fever as a model, symptoms developed between the second and fifth day after infection, associated with the typical leukopenia. The quantitative nitro-blue tetrazolium dye reduction test showed a significant decrease in activity of the individual cells between the fourth and seventh day. There was also a reduction in glucose-6-phosphate dehydrogenase activity but there was no alteration in the leukocyte 6-phosphogluconic dehydrogenase activity. In addition, there was a defect in the bacteriocidal activity of the leukocytes as measured by their effect on Staphylococcus aureus. The earliest changes and greatest amplitude occurred in those with the most serious illness. The bacteriocidal deficiency of the neutrophils occurred between the seventh and the twenty-fifth day after inoculation of the viral challenge. Although this suggests a greater susceptibility to bacterial infections this has not been a feature of sand fly fever. In working with measles, Dr. Bellanti was able to develop a quantitative test of cell-mediated immunity based on chromium-51 release from a tissue culture system of Hela cells or WI-38 cells chronically infected with measles virus. Lymphocytes from individuals with demonstrable antibodies destroyed a cell sheet of measles infected cells within two days, or caused the release of chromium-51 from infected cells within 4-6 hours. Individuals with previous natural measles released approximately 5-6 per cent of their chromium-51, cases with intercurrent infection up to 16 per cent. Of particular interest was the fact that sera from patients with subacute sclerosing panencephalitis have high serum neutralizing titers but very low chromium-51 release. The interpretation of this observation afforded an opportunity for fascinating hypotheses but no conclusions.

f. Dr. Noel R. Rose (Cellular and Humoral Immunity in Parasitic Infections) has continued his work on immunity to the parasitic infection toxoplasmosis. In the guinea pig, evidence of delayed hypersensitivity could be elicited by the injection of toxoplasma antigen in complete Freund's adjuvant or by establishing a nonlethal prolonged infection with virulent organisms. Circulating antibody was demonstrated by the indirect immunofluorescent antibody technique (IFA). The correlation between circulating antibody and macrophage inhibition was excellent, with a coefficient of correlation of about 0.85. A blastogenic response to toxoplasma antigen in lymphocyte culture could be determined by uptake of tritiated thymidine. Here, there was an inverse correlation between the levels of circulating antibody and the amount of thymidine uptake, and in addition, the lymphocytes of some uninfected guinea pigs with no demonstrable antibody underwent blast transformation. With peritoneal exudate cells, a nonantigen-specific stimulation of their ability to ingest and destroy toxoplasma resulted from the injection of complete Freund's adjuvant. The spleen and lymph cells are specific in their reaction. Toxoplasma become attached to the surface of lymphocytes and are killed by a lymphocyte-mediated and probably antigen-specific reaction. Using rats, the establishment of a toxoplasma infection diminished or inhibited the formation of plaque-forming cells after intravenous injection of sheep red blood cells. In the discussion, those portions of the protective mechanisms which are attributable to cellular immunity and those to humoral immunity are yet to be differentiated.

g. Dr. G. Edsall (Factors Influencing the Pattern of the Immune Response)attempted to define the factors which determined those individuals who were protected against tetanus by a single dose of tetanus toxoid. Unfortunately, the study in

Louisiana finally contributed too few individuals to clarify the issue. Some elderly people were excellent responders; in the small numbers available, there was no clear indication that the female was more responsive than the male. There was a suggestion of possible family relationships, but the groups were too small to justify any conclusion that the response is genetically established. In attempting to develop a diphtheria toxoid with greater antigenicity, the interesting observation was made that when glutaraldehyde aggregation was terminated by sephadex filtration, the resulting material elicited in guinea pigs a hemagglutinating response, principally in the 7S fraction. When the aggregation was terminated by the addition of lysine, the hemagglutinating response was greater and was primarily a 19S response; however, this antigen afforded a much less effective protection to the guinea pig against a toxin challenge. This reaffirms the need to actually assay protection, rather than antibody response, from immunizing products. With tetanus toxoid, column purification of the toxin before toxoiding with formalin resulted in a fluid toxoid approximately ten times as antigenic as the usual ammonium sulphate toxoid; the antigenicity of this toxoid was not enhanced significantly by absorption on aluminum salts.

In the discussion of this work, Dr. Pappenheimer cited current investigations on diphtheria in his laboratory. Using a variety of mutants of the lysogenic phage, they have found one which provides structural information for synthesizing a molecule which apparently differs from diphtheria toxin in a single amino acid substituted in the enzymatically active portion; this product is physiologically inactive but antigenically identical to diphtheria toxin. This approach suggests the possibility of biologically developing chemical analogues of toxins which would provide antitoxic immunity without the current problems of degraded antigenicity from excessive denaturation, or the reversion problem which is being experienced in developing a cholera toxoid.

h. Dr. K. F. Meyer (Isolation and Characterization of Lymphogranuloma Venereum Agents) was unable to attend the meeting, but was represented by Dr. Julius Schachter. Their group has been studying cases of lymphogranuloma venereum, primarily among servicemen or merchant seamen who acquired their infection in South East Asia. Thirty-two clinical cases have been investigated. The classical diagnostic technique, the Frei test, proved to be positive in only 37% of the cases tested. Isolation of the agent was only accomplished in nine of the thirty cases tested; this is explained by the fact that chemotherapy had often been initiated before the specimens were obtained: an agent has not yet been isolated from a patient who has had as much as one day of chemotherapy. Fifty-six per cent were positive in the complement fixation test. Investigation of the Frei test by testing laboratory-infected monkeys and rabbits with a variety of laboratory prepared antigens revealed a great deal of erraticism. Some animals did develop a hypersensitivity when tested over a long period of time; others remained negative while all animals were serologically positive. Studies with the complement fixation test disclose the interesting observation that the complement fixation test on whole serum from laboratory infected African green monkeys could be negative for up to four weeks following infection; however, when these sera were column-fractionated, there were good titers in the IgM fraction within the first week after infection, indicating the presence in the acute phase serum of an inhibitor. Whether this applies in human sera has not yet been established. The agents isolated to date have been characterized into three different antigenic groups with close relationship to the trachoma-inclusion conjunctivitis organisms. The importance of this group of organisms in the etiology of non-specific urethritis is under scrutiny. Among sera obtained in Vietnam, 15 to 25% have complement fixing antibodies at a 1:8 or greater level, not different significantly from the frequency found among sexually active men in San Francisco. In the San Francisco area, 25-30% of Reiter's syndrome cases were found to be infected with chlamydia. The studies on the involvement of this group of organisms in the entity of non-specific urethritis continues.

i. Dr. H. N. Eisen (Antibody Structure and Function) was unable to attend the meeting. He has continued his work in defining the point on the antibody molecule to which the antigen attaches. The complete sequence of 214 amino acid which constitutes the light chain of myeloma protein 315 has been defined and it is the tyrosine residue in position 34 which binds bromoacetyl-Dnp ethylenediamine (BADE). It is pertinent that, in its hyper-variable region, this antibody which is anti-Dnp in its activity, differs in only 8 of the 110 residues from the light chain from protein 104 which has no anti Dnp activity and is anti-dextran in its activity. The heavy chain of protein 315 bears a lysine residue at position 54where the protein binds BADL. A specially fabricated bifunctional affinity-labeling reagent with two bromoacetyl groups was bound specifically by protein 315 and covalently crosslinked the tyr-34 of the light chain and lys-54 of the heavy chain, demonstrating that the hypervariable regions of light and heavy chains are within 5Å of each other. The active combining sites of the antibodies lie in the hypervariable regions.

When mice were immunized with these myeloma proteins antibodies were formed which reacted only with the unique idiotypic determinants which were apparently located at these combining sites, since Dnp ligands block the reaction. When these immunized animals were inoculated with the myeloma tumor cells which had formed this protein, there was a relative resistance to tumor formation and when tumors did occur, they no longer secreted protein 315.

j. Dr. N. R. DiLuzio (Role of the Reticuloendothelial System and Humoral Recognition Factors in Malaria Infection) was unable to attend the meeting. He has shown that chromium labeled erythrocytes infected with Plasmodium berghei are removed from the blood at a significantly accelerated rate over normal non-infected erythrocytes. Neither stimulation nor depression of the reticuloendothelial system altered the phagocytic clearance of the labeled erythrocytes. Stimulants such as diethylstilbestrol and melezitose did however significantly reduce parasitemia and mortality. There was no evidence that the antimalarial drugs quinacrine or chloroquine have a functional synergism with the reticuloendothelial system. Antilymphocytic serum given intraperitoneally had no significant effect on the degree of parasitemia nor on mortality; intravenously it did have a profound enhancing effect on the degree of parasitemia. Stimulation of the RES is usually associated with endotoxin hypersensitivity and in fact malaria infected mice were found to be approximately 40-fold as susceptible to endotoxin as normal mice. Liver homogenates from malaria infected donors had lost their endotoxin detoxification ability. This is presumed to be the consequence of disturbance of the function of the Kupffer cells and not of the parenchymal cells. Since endotoxin sensitive animals are reported to have an increased bacteriocidal activity, peritoneal macrophages from malaria infected mice were incubated with Staphylococcus aureus and indeed a significantly enhanced bacteriocidal activity was demonstrated when compared to the peritoneal macrophages obtained from non-infected mice.

k. Dr. W. F. Verwey (Responses of Humans to Cholera Vaccine Immunization) did not report to the meeting pending the completion of the scheduled time intervals for bleedings and antibody studies. To date, his data indicates that the response to a booster dose two years after initial two dose immunization is as good as that at six months or one year. Although initial immunization with two doses one week apart results in a better mean response than two doses four weeks apart, both groups responded equally well to vaccine two years later. When the responses to a standard vaccine are compared to those to an Inaba monovalent vaccine which had been shown to produce a high degree of protection after a single dose in Pakistan field trials, the vibriocidal antibody responses were equivalent or superior.

III. Review of Areas of Concern of the Commission

a. Gammopathies

Sera have been collected from personnel at Ft. Detrick who had been subjected to immunization with a large number of antigens but not with cholera vaccine, and from Canadian-Pacific Airline personnel who have been repeatedly immunized, including cholera vaccine. Their cooperation was obtained because of the frequent reactions reported by these individuals after cholera immunization. Serological studies are underway to define the character of immunoglobulins present in these sera.

b. Smallpox

Dr. Benenson reported on the progress of four universities evaluating the effect of reduced dosage and different viral strains on reactions. The recent Public Health Service recommendation that routine smallpox vaccination be discontinued within the United States may have an adverse effect on the completion of these studies.

c. B. Virus

The hopes for availability of a B virus vaccine have not yet been realized. The problem lies in the necessity that all virus be inactivated and the ease with which antigenicity is lost. The lot prepared for our use proved of inadequate antigenicity, and we are awaiting a lot which had been adequately tested for safety and still retains antigenicity.

d. Rabies Vaccine

Dr. Gochenour reported progress in the development of tissue culture vaccines. A subunit product has been prepared which in monkeys has proved to be superior to current vaccine. In the Habel mouse test where a vaccine is considered to have adequate potency when it has an index of 0.3 compared to DBS reference vaccine, the new vaccine has an index of 30 to 100. Field trials on this vaccine are to begin soon.

e. Mumps

The Air Force representative raised the question of the relative importance of mumps in the Armed Forces at present. In 1970 they had an incidence rate of 52.7 per 100,000, in 1971, 69.5, resulting in 3889 days lost from duty in 1970 and 2412 days in six months of 1971. There were 168 cases of orchitis in 1968; 105 in 1969, but the number of complications since this time are not available because of a change of coding of data. These rates may well justify consideration of administering mumps vaccine to those who deny having had this disease.

IV. Accomplishments during the Year

Significant progress has been made in several areas of concern to the Commission on Immunization.

a. In developing an understanding of the molecular phenomena involved in immunity, the amino acid sequence of the light chain of one antibody has been completely defined, and its antigen attaches to the tyrosine residue at the 34 position. Light chains from two proteins with entirely different antigenic specificity differed only in eight of the 110 residues in the hypervariable region, where the active combining sites are found. The antigenic receptor site on the light chain and that on the heavy chain in the intact protein are physically within five angstroms of each other.

b. Studies on small lymphocytes have shown that IgM antibody is physically present as a 7-8S monomer on the surface of the small lymphocytes of the B type, i.e. precursors of cells which will secrete predominantly IgG antibody. A model was proposed for the control of antibody level, i.e. that the stimulus for antibody synthesis related to the antigen: antibody ratio. The synthesis of IgE in mice after small antigen doses is controlled by a single autosomal locus closely linked to the H-2 type; the relation is antigen-specific. With a high dose of antigen, some mouse strains produce only IgG and others produce both IgG and IgE; this response, which is not antigen-specific, appears to be a multi-locus effect. Homocytotropic antibodies can be elicited in both guinea pigs and rabbits by repeated small doses of antigen in aluminum gel. Studies on the ontogeny of the immune response suggests that IgA can be produced early during fetal life in situ when an antigenic stimulus is afforded. Immunological maturity to specific antigens occurs independently of the development of secondary germinal centers.

c. The typhoid model of Salmonella enteritidis in the mouse reaffirms the minimal protection that follows the administration of killed vaccines. However, with challenge strains of low virulence, there can be demonstrated some immunity which involves a delay or inhibition in the spread of organisms from the gut into the systemic circulation, as well as increased destruction of the organisms which do reach the spleen and liver.

d. A viral infection (sand fly fever) produces a marked reduction in glucose-6-phosphate dehydrogenase activity in neutrophils and defective bacteriocidal activity (using Staphylococcus aureus). A technique for a quantitative measurement of cellular hypersensitivity has been developed with measles virus, disclosing the interesting paradox that patients with subacute sclerosing panencephalitis (caused by measles virus) have high serum neutralizing titers but very low levels, if any, of cellular hyperimmunity.

e. With parasitic infection, studies on toxoplasmosis and on malaria are underway to differentiate the relative effects of humoral and cellular antibody. In toxoplasmosis, macrophage inhibition and blastogenic responses could be quantitated. Direct visualization of toxoplasma discloses a lymphocyte-mediated and probably antigen-specific reaction. The tools to define the relative importance of humoral versus cellular immunity are being firmly established. Malarial infection in mice is associated with a markedly increased hypersensitivity to endotoxin. This was associated with significantly enhanced bacteriocidal activity in the peritoneal macrophages from malaria-infected mice.

f. Different methods of production of diphtheria and tetanus toxoids had a large effect on the type of antibody response and protection elicited. Hope is held out for the development of immunizing preparations against diphtheria by the use of a lysogenic phage (which controls the elaboration of diphtheria toxin from Corynebacter diphtheriae) which elaborates a slightly modified product which is fully antigenic, but devoid of toxicity.

8. Studies on lymphogranuloma venereum have confirmed the unreliability of the Frei test, even when a homologous antigen is being used. The complement fixation test on laboratory-infected monkeys revealed the paradox of that while whole serum was negative up to four weeks, positive reactions were obtained within the first week after infection with the IgM fraction. Whether this explains CF failures in man has not been established yet.

The joint meeting with the Commission on Epidemiological Surveys was of great benefit to the members of the Commission on Immunization in appreciating some of the practical problems as well as having the opportunity to become familiar with the immunological problems which are being confronted by other groups of the AFEB.

The Director wishes to thank the members of the Commission and the staff of the AFEB for their cooperation and support.

Respectfully submitted,

Abram S. Benenson, M.D.
Director
Commission on Immunization

ASB/mb

PHYSICOCHEMICAL STUDIES AND BIOLOGIC SIGNIFICANCE OF ANTIVIRAL ANTIBODY

ANNUAL REPORT

by

Joseph A. Bellanti, M. D.

(For the period of 1 July 1969 to 30 June 1971)

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Washington, D. C. 20315

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Georgetown University School of Medicine
Washington, D. C. 20007

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ABSTRACT

1. Preparing Institution: Georgetown University School of Medicine
2. Title of Report: Physicochemical Studies and Biologic Significance of Antiviral Antibody
3. Principal Investigator: Joseph A. Bellanti, M. D.
4. Number of Pages, tables and figures: Pages 20, tables 16, figures 2
5. Date: June 1, 1971
6. Contract Number: DA-49-193-MD-2633
7. Supported by: U.S. Army Medical Research and Development Command

This contract continues to be a study of the biologic significance of antiviral antibody in order to assess its role in immunity in the human. Control mechanisms and sequence data are being sought. The study of serum and local antibody responses has continued and has been performed in children immunized with live rubella vaccine.

The studies have been extended to include two other major areas concerned with antiviral immunity: 1) studies of biochemical and biologic events involved in phagocytosis, and 2) studies concerned with cell-mediated (delayed hypersensitivity) immunity to measles virus.

Key words: Virus, antibody, immunoglobulins, immunization, radioimmunodiffusion, phagocytosis, cell-mediated immunity, delayed hypersensitivity.

I. Introduction

This report describes the results obtained during the sixth year of studies of the biologic significance of antiviral antibody. The overall objectives of these investigations have been to define the biologic significance of various immunochemical classes of antibody resulting from immunization or infection. These studies have dealt with the role of these antibodies in protective immunity.

During the sixth year of studies, essentially every category of endeavor outlined in the original proposal has received attention. In addition, two new lines of investigation have been explored. The first includes the biochemical and biologic events concerned with phagocytosis in the normal and during conditions of induced experimental viral infection. The second major new area is the development of an in vitro test for detection of cell-mediated immunity (delayed hypersensitivity) to measles virus employing a lymphocyte target cell interaction with a chromium radioactive release.

Results are given in four major areas:

A. Physicochemical studies of humoral and local antibody responses to immunization with viral vaccines.

B. Biochemical and biologic studies of phagocytosis.

C. Studies of cell-mediated immunity to measles virus.

D. Other studies related to the basic proposal supported by the contract.

II. Personnel

The laboratory and clinical studies supported by the U.S. Army Medical Research and Development Command under Contract No. DA-49-193-MD-2633 have been concerned primarily with the biologic significance of antibody. A number of individuals and agencies not directly associated with nor supported by this contract have collaborated in many phases of this work. Their contributions are acknowledged in the body of this report. Although all of the following personnel are not supported directly by this contract, their participation in the work reported is gratefully acknowledged.

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III. Research Results

A. Physicochemical studies of humoral and local antibody responses following immunization with viral vaccines.

Over the past year, a major area of effort of this laboratory has been a continuation of studies of local and serum antibody responses following immunization with viral vaccines. This is an extension of investigations concerned with elucidating factors important in host resistance. Responses have been studied following the use of live rubella vaccine. This viral disease known to be a cause of morbidity in military recruits is now being eradicated through the use of rubella vaccine.

1. Serum and local antibody responses in children following immunization with attenuated rubella vaccine.

Subjects. Ten rubella seronegative children were immunized with live rubella vaccine (Cendehill strain, live rubella virus vaccine). Each child received 0.5 ml. subcutaneously.

Collection and processing of specimens. Blood and nasal secretion specimens were collected from each of the children prior to and at two week intervals following vaccine administration for a total of 14 weeks. The sera were collected aseptically and stored frozen until testing. The methods used for collection and processing of nasal secretions have been described previously (Annual Reports 1965-66; 1966-67).

Antibody tests. Serum antibody was determined by the hemagglutination-inhibition (HI) method using kaolin extraction. Since this method was found to be unsatisfactory for the detection of antibody in nasal secretions, the interference method using ECHO 11 challenge was employed for the determination of neutralizing antibody in nasal wash specimens.

Results. The development of serum HI antibody to rubella virus is shown in Table I. It can be seen that antibody is detected as early as the 14th day after immunization in 3 of 10 children and in all subjects by the 28th day. The antibody responses in serum appear to be heterogeneous, ranging from maximal titers of 1:16 in subject #5 to titers of 1:2048 in subject #7.

The results of nasal antibody responses are given in Table II. Antibody is detected as early as the 14th day in 4 of 8 specimens, in 8 of 9 by the 28th day, and in all 9 by the 56th day. The antibody responses in nasal secretions were also heterogeneous.

Conclusions. These results indicate that serum and nasal antibody develops following the parenteral administration of live rubella vaccine in these subjects. This response is similar to that described for other live viral vaccines such as rubeola vaccine (Annual Report 1967-68). With the evidence at hand suggesting a protective role for local antibody, it seems reasonable to assume a similar protective role for the antibody engendered by this vaccine. Studies are currently in progress to develop a radioimmunoelectrophoretic method for the complete characterization of antibody in serum and in nasal secretions. These studies employing radioactively labeled C^{14} rubella virus will be continued over the coming year's support. In addition, studies are contemplated employing live rubella virus vaccine administered intranasally. Both nasal and serum antibody responses will be tested following this approach. These results will be compared to those obtained following the use of vaccine administered subcutaneously.

Since 1964, the overall objectives of this contract have been the elucidation of control mechanisms important in antimicrobial immunity. In keeping within this general framework, a major portion of this laboratory's efforts have been directed to the area of cellular immunity. These have included two major areas: 1) studies of phagocytosis, 2) studies of cell-mediated (delayed hypersensitivity) responsiveness.

B. Biochemical and biologic studies of phagocytosis.

Background: The immunologic system may be viewed as a collection of cells and cell products concerned with the recognition of foreigness. The response of the host to microbial agents is but one example of the reactivity of the immune response to foreigness.

In dealing with microbial agents, the cells that are most importantly involved in first encounter with a microbial agent are the phagocytes (macrophages and polymorphonuclear leukocytes). In general, there is agreement that the process of phagocytosis is a multiplex process involving the participation of these phagocytic cells and humoral factors (antibody and complement). Deficiencies may occur either in the cellular participation as in the young infant or in the inborn errors of metabolism affecting leukocytes (chronic granulomatous disease). Deficiencies may also involve complement or antibody as seen in certain other types of deficiency states.

Recent studies of the biochemical events concerned with phagocytosis have shed considerable light on the process. During phagocytosis the energy requirement for particle uptake and subsequent intracellular digestion is derived from glucose. Glucose may be metabolized either by the Embden-Meyerhof pathway or through the hexose monophosphate pathway (HMP). It is now recognized that during intracellular digestion the HMP increases its activity from approximately 5% in the resting state to 40% during the phagocytosing state. Clearly, the HMP is a key pathway for the leukocyte as it has been shown for the erythrocyte. The eventual killing of bacteria is thought to be related to this enhanced HMP activity through the generation of hydrogen peroxide which is thought to be the actual bactericidal mechanism. Reflective of this enhanced HMP activity during phagocytosis is the increased flow of protons which can be easily measured by the reduction of the dye nitroblue tetrazolium (NBT) from its colorless form when oxidized to the deep blue form in its reduced state. The studies to be described are directed particularly at studies concerned with the activities of the HMP with particular reference to the first two enzymes of the shunt: 1) glucose-6-phosphate dehydrogenase (G-6-PD) and 2) 6-phosphogluconic dehydrogenase (6-PGD).

These studies of phagocytosis were prompted by the rapidly accumulating evidence that defects of phagocytosis may provide important biologic markers of immunity which could readily detect individuals most at risk to infection. Secondly, these studies are directed at obtaining specific information regarding the maturation of these processes within the human as well as the changes which occur during the course of acute viral infections. Accordingly, the studies to be described are directed at obtaining information in the following areas: 1) studies of phagocytic function in individuals predisposed to infection (chronic granulomatous disease) and 2) studies of leukocyte function during experimental Sandfly fever virus infection in the human.

1. Studies of individuals predisposed to bacterial infection: chronic granulomatous disease (CGD).

Chronic or fatal granulomatous disease (CGD) was originally described as an X-linked recessive disorder affecting children and manifested by recurrent infections with organisms of low virulence. Histopathologic changes in affected organs consist of granulomatous infiltrations containing plasma cells, lymphocytes and macrophages. The leukocytes of affected patients can ingest bacteria but are apparently unable to digest them.

A metabolic basis for the defect is suggested by the inability of phagocytosing leukocytes to develop the characteristic increase of respiration, hexose monophosphate shunt (HMP) activity and hydrogen peroxide formation occurring in normal leukocytes during phagocytosis. Leukocytes from affected patients to reduce the oxidized form of nitroblue tetrazolium dye (NBT) during phagocytosis.

It has been suggested that the inherited defect might be due to diminished activity of an enzyme, NADH oxidase which indirectly promotes increased HMP activity during phagocytosis via an NADPH-linked lactate dehydrogenase. Yet in species such as the rat, for example, lacking NADPH-linked lactate dehydrogenase in polymorphonuclear leukocytes such a mechanism cannot account for the augmented oxidation seen during phagocytosis. Therefore, we decided to search further for the molecular basis of CGD. It will be shown that an abnormally rapid rate of decay of glucose-6-phosphate dehydrogenase (G-6-PD) activity occurs in the leukocytes and red cells of patients with CGD.

The approach to the problem:

Three children with classical findings of CGD were studied at Walter Reed General Hospital and Georgetown University Hospital (Table III). All subjects tested were shite males. Age-adjusted and adult controls were also included for comparison.

Methods for the preparation of a relatively rich suspension of granulocytes were those described by Baehner and Nathan with slight modifications. Thirty mls. of heparinized blood were obtained from each subject. Sedimentation was facilitated by adding sufficient 10% dextran in 0.15 M NaCl to each specimen to obtain a final concentration of 1% dextran. Following removal of the leukocyte-rich plasma, two volumes of 0.87% ammonium chloride were added to the plasma to remove any residual erythrocytes. Centrifugation at 100 g was performed for 5-10 minutes. The supernatant was withdrawn and the cells were washed twice with Krebs-Henseleit-bicarbonate buffer, pH 7.4 containing 200 mg% of glucose. Differential counts of this fluid revealed values consistently in excess of 90% polymorphonuclear leukocytes.

Cell homogenates were prepared either from the frozen crude cell suspension or from fresh cells without freezing. Homogenization was accomplished either by repetitive freeze-thawing or by ultrasonication. Immediately after cell disruption the homogenates were centrifuged at 4°C for 15 minutes at 20,000 g in the Spinco Model L-2 ultracentrifuge. The supernatants were analyzed for protein content by the Lowry method and the enzymatic assays were performed as described below.

The activities of G-6-PD and of 6-phosphogluconic dehydrogenase (6-PGD) in red cell and white cell lysates were determined as described by Richterich. The results of leukocyte enzyme activities were measured in International units (IU) as umole/min/gm of protein at 25°C and are expressed as % activity of that measured at time 0. Red cell G-6-PD activity was similarly determined on hemolysates of blood collected in acid citrate dextrose (A.C.D.) solution. Results were measured in IU as μmole/min/gm hemoglobin at 25°C and are expressed as % activity of that measured at time 0.

Experiments were also performed to determine the effects of storage, stabilizing agents and temperature on G-6-PD activity. Leukocyte homogenates prepared from leukocytes frozen at -70°C in the presence of 10 μM NADP, 14 mM 2-mercapto-ethanol (2-ME) or 10 uM NADP plus 14 mM 2-ME were tested at 0, 4 and 18 hours following storage at 4°C.

Comparable results were obtained when the various agents were added to fresh cells without prior freezing. Measurements of red cell activities were performed similarly. After preparation of red cell hemolysates NADP or NADP and 2-ME were added to the hemolysates in tests of stability as described above. Thermostability of G-6-PD was determined by heating the homogenates for 0, 10, 20 and 30 minutes at 38°C prior to testing.

Electrophoretic mobility was determined in polyacrylamide gel and in starch gel (Tris HCl, pH 8.8 buffer). Michaelis constants (Km) were determined for G-6-PD using glucose-6-phosphate as substrate.

The quantitative NBT test was performed by the method of Baehner and Nathan results being expressed as the ΔOD between resting and phagocytosing values for 2.5×10^6 cells/15 minutes at 37°C. The bactericidal assay was performed employing Staphylococcus aureus or Escherichia coli and the results expressed as % reduction in viable bacteria after 120 minutes of incubation with polymorphonuclear leukocytes.

Results:

All patients displayed an inability to reduce NBT dye and had impaired leukocyte bactericidal activity (Table III). Preliminary testing revealed diminished leukocyte G-6-PD activity in all 3 patients when compared to age-related or adult controls.

Since there appeared to be wide fluctuations in the G-6-PD activity of patient leukocytes from one determination to another suggesting enzyme lability, the effect of storage was next studied (Table IV). It may be seen that the rate of decay of leukocyte G-6-PD activity in each of the 3 patients exceeded that seen in age-related and adult controls. Assays were also performed on homogenates of fresh unfrozen cells in patients S.E. and B.P. In both, a similar rapid decay was observed following storage. In all 3 patients the red cell G-6-PD activities at 0 time were similar to those of controls (Table IV). By 18 hours, however, a more rapid rate of decay was observed in patients with CGD than in normal subjects.

The effects of stabilizing agents on leukocyte and erythrocyte G-6-PD activities are given in Table V. It may be seen that although a stabilization effect was observed in leukocyte homogenates from normals as well as patients with 2-ME or 2-ME plus NADP, the effect was less marked in patients. A stabilizing effect of NADP was observed only in controls and not in patients with CGD. In erythrocytes, stabilization

was observed with NADP and 2-ME in both patients and controls to approximately the same degree. The thermostability of leukocyte and erythrocyte G-6-PD at 38°C in 2 patients and a control is shown in Table VI. A marked lability was observed in leukocytes and erythrocytes in each of the patients when compared to control values. The results of electrophoretic analyses and determination of Michaelis constant (Km) of leukocyte G-6-PD were essentially the same in patients and in controls.

The activity of a second NADP-dependent enzyme, 6-PGD, was studied in leukocyte homogenates and results are given in Table III. It can be seen that the activities in patients were comparable to those in age-related and adult controls.

The relationship between the quantitative NBT test and G-6-PD activity is shown in Figure 1. Data are included on infants and children of varying ages as well as patients with CGD and individuals of negro and middle eastern origin. It can be seen that a linear correlation exists between the two variables with a correlation coefficient of 0.86, $p < .01$.

Discussion:

The results of the present studies reveal a more rapid rate of decrease in glucose-6-phosphate dehydrogenase (G-6-PD) activity in the leukocytes and erythrocytes of patients with CGD than in normals. Several hypotheses may be considered to explain these findings. The first is that there exists in CGD an increased activity of an enzyme degradative system or some other influence on a normal G-6-PD protein such as an inactivator or inhibitor. Leukocytes are known to possess relatively high concentrations of proteolytic substances which can affect G-6-PD activity. The more rapid fall in G-6-PD activity during storage in leukocytes compared with the relatively slow rate in red cells is consonant with this view. However, red cell activity decreased more rapidly in patients with CGD during storage and with heating than in the normal, suggesting that the enzyme itself might be more vulnerable to normal degradative systems. The stabilization effect of 2-ME on the G-6-PD of patients and controls, underscores the importance of maintaining critical sulfhydryl groups on the protein.

Another hypothesis is that of a variant G-6-PD protein. The mode of inheritance of most cases of CGD has been shown to be X-linked, corresponding with the established genetic locus for G-6-PD. The finding of diminished activity of enzyme in red cells and white cells of patients is consonant with this hypothesis. Further, the observation that NADP alone does not stabilize the G-6-PD enzyme of patients, may indicate an abnormal binding of NADP. An abnormality in G-6-PD activity would also explain the diminished ability in CGD of leukocytes to reduce NBT dye. A high degree of correlation was found between leukocyte NBT reduction and G-6-PD activity (Figure 1).

The disease has recently been described in females suggesting the possibility of genetic heterogeneity for the entity with some cases resulting from an autosomal mutant gene. The pedigree data and phenotypic expression in females are also compatible with variable degrees of expression by an X-linked gene due to incomplete dosage compensation. Although this issue is unresolved at present it is not unlikely that a number of different genetic mutations and molecular disorders of metabolism will be found to underly the clinical entity now recognized as CGD.

The importance of CGD is that it represents the first model of an inborn error of metabolism affecting the leukocyte. This defect presents with an increased frequency to bacterial infection. It provides a handle with which to study the increased susceptibility in the normal human. The findings of a labile G-6-PD have directed our attention to studies of the maturation of this enzyme in the human during development and also during experimental viral infection.

2. Studies of leukocyte function during experimental Sandfly fever virus infection in the human.

The second major area of study of the phagocyte has been performed in volunteers experimentally infected with Sandfly fever virus. These studies have been performed in collaboration with Dr. Beisel, Lt. Bartelloni and Col. Dan Crozier at the Medical Unit, Ft. Detrick, Maryland.

The problem:

To gain additional information concerning neutrophile function during the course of experimental infection with Sandfly fever virus.

Background:

These studies arose out of a need for developing a suitable marker for the detection of individuals at risk to infection. It is now well known that following many types of viral infection, there is a susceptibility to bacterial infection. Following influenza, for example, secondary infection with Staphylococcus is a frequent occurrence. This is often the cause of serious and sometimes fatal pneumonia. Surprisingly little is known of the biochemical and biologic events within the phagocyte concerned with these findings.

Sandfly fever is a disease caused by an agent belonging to a group of arboviruses which in nature are disseminated by the vector *Phlebotomus papatasi*. The clinical manifestations of the disease have been best elucidated from studies of the experimental disease in volunteers. Following parenteral administration of the virus, the length of the incubation period is dependent upon the route of inoculation; following intracutaneous inoculation, 95% of the volunteers developed the disease after 2 1/2 to 6 days in contrast to 44 hours when the virus is administered intravenously. The disease is characterized by the sudden onset of systemic findings of anorexia, malaise, headache and fever lasting 1 - 3 days. The striking laboratory feature in this disease is a *leukopenia* occurring at the end of the febrile period and consisting of a lymphopenia and a corresponding increase in neutrophils (mainly immature forms).

Approach to the problem:

These studies were performed in collaboration with the Fort Detrick group as part of their ongoing studies concerned with metabolic changes occurring during the course of induced experimental infections.

Virus inoculum:

The Sicilian strain of Sandfly fever virus was used in these studies. It was obtained from the plasma of a volunteer who had been experimentally infected with the virus and whose plasma was shown to be infectious.

Subjects:

Six volunteers received 0.5 ml. of plasma intravenously; 2 subjects served as controls and received 0.5 ml. of normal saline intravenously.

Specimens:

Twenty (20) ml. to 30 ml. of heparinized blood was obtained from each subject on days -3, -1, +1, +3, +4, +7 and +19.

Biochemical determinations:

The quantitative NBT test , G-6-PD and 6-PGD enzyme determinations were performed according to methods described in a preceding section of this report. The quantitative NBT dye results are expressed as the ΔOD between resting and phagocytosing values for 2.5 x 10^6 cells/ 15 minutes at 37°C; the G-6-PD and 6-PGD as muM NADPH reduced/minute/mgm of protein.

Results:

The results of the quantitative NBT tests are given in Table VII. It can be seen that in each of 6 subjects undergoing infection, a decrease in quantitative dye reduction was observed. This occurred between the 4th to the 7th day and levels as low as 20% of baseline values were observed. In contrast, similar changes were not observed in the controls. The reduced values all returned to normal by the 19th day.

The changes in leukocyte G-6-PD and 6-PGD activity are given in Tables VIII and IX. A reduction in G-6-PD activity was observed in each of the 6 volunteers undergoing experimental infection. This was usually detected by the 7th day (Table VIII). Activities as low as 5 - 6% of baseline values were observed in some of the men in contrast to the controls who failed to show similar changes. G-6-PD activities did not return to baseline values in 5 of 6 men for as long as 19 days, the last day of testing. The activities in the second enzyme of the HMP, 6-PGD did not show changes in either controls or infected subjects (Table IX).

Discussion:

The results of these studies indicate that there are measurable in vitro changes which occur within the neutrophile during the course of experimental viral infection. These consist of changes in NBT dye reduction and G-6-PD function, variations indicative of impaired HMP activity. These changes appear to be valid since each of the measurements were standardized to a fixed number of cells or to a fixed protein concentration. These results of these studies are now being compared and correlated with other changes occurring during the course of this

type of infection. These studies will be extended over the next year to include studies of changes in bactericidal capacity (employing Staphylococcus aureus) during the course of experimental infection with Sandfly fever virus. It is hoped that these studies will extend further our knowledge concerning the causes for increased susceptibility to infection in the human.

C. Studies of Cell-Mediated Immunity to Measles Virus

1. Development of in vitro techniques for measurement of cell-mediated immunity.

The problem.

To develop a method for measurement of cell-mediated immunity to measles virus in the human.

Background.

It is now generally accepted that there exists two types of effector mechanisms concerned with the elaboration of specific immunity: 1) a humoral mechanism in which antibody is involved and 2) a cell-mediated (delayed hypersensitivity) mechanism which occurs independent of antibody and which is mediated through the action of specifically sensitized lymphocytes. The cell-mediated response is currently the focus of great medical interests because of its role in the rejection of grafts, its role in the pathogenesis of autoimmune disease and as an important surveillance mechanism implicated in the prevention of neoplasia. The delayed dermal reaction characteristic of the in vivo manifestations of cell-mediated immunity have long been recognized following many types of bacterial, viral, and fungal infections. Its precise quantitation in vitro, however, has only been recently accomplished.

Studies of viral immunity have been largely restricted to the study of antibody mediated events. There has been a curious absence of studies related to the cell-mediated (delayed hypersensitivity) mechanisms of immunity. There is now accumulated evidence that this mechanism may be a most important mechanism of immunity in the human, particularly with viruses which mature at cell surfaces (myxo-, paramyxoviruses, vaccinia, herpes simplex). Because of its potential importance as a valuable marker of immunity in the human, a major effort of our laboratory has been devoted to the study of this mechanism.

Following interaction of antigen with a specifically sensitized lymphocyte there is released a number of soluble factors which are the effectors of the cell-mediated reaction. These are listed below:

Possible Mediators Produced In Vitro by Lymphocytes Stimulated with Antigens

Substance	M.W.
Migration Inhibitory Factor (MIF)	70,000
Skin Reactive Factor	70,000
Lymphotoxic Factor (LT)	80,000
Cloning Inhibiting Factor	
Chemotactic Factor	60,000
Blastogenic Factor	
Interferon	(25,000)
Cytophilic Antibody	140,000

Macrophage inhibitory factor (MIF) is a factor which prevents the migration of macrophage cells. A skin reactive factor causes a type of reaction characterized by induration and erythema upon inoculation into the skin of normal guinea pigs. A cytolytic factor called lymphotoxic factor (LT) has been shown to suppress protein synthesis in a variety of target cells from many species and ultimately causes cell death. This has been conveniently measured by the release of radioactive chromium^{51}. A cloning inhibiting factor is also released which blocks the ability of hela cells to divide thereby drastically limiting the number of clones one is able to detect. A chemotactic factor which is involved in the attraction of phagocytic cells and a blastogenic factor which stimulates mitosis are other factors which are released upon the interaction of antigen with specifically sensitized cells. Interferon and antibody have also been shown to be released after this interaction.

Approach to the Problem.

Materials and Methods.

Lymphocytes were obtained from individuals recently immunized with measles vaccine, individuals undergoing natural measles infection, or individuals who gave a negative history of infection or immunization. Approximately 20 ml. of heparinized blood were allowed to sediment by gravity and the leukocyte-rich plasma layer was removed. The neutrophils were removed by passage through glass bead columns 1 X 20 cm. and purified lymphocytes were obtained. The lymphocyte suspensions

were washed two times in balanced salt solution and adjusted to a final concentration of 1 X 10^6 ml.

Target cells.

Tissue culture lines of HeLa or WI38 chronically infected with measles virus were obtained from Dr. Robert Rustigian. These cells were shown to be infected with measles virus by means of immunofluorescence or direct hemagglutination employing rhesus monkey erythrocytes. Using these techniques, 80 to 90% of the cells were shown to contain measles virus.

Measurement of reactivity.

Two types of measurement were employed for these studies: 1) a cytologic technique in which lymphocytes were added to monolayers of control and infected cells and cytopathogenicity (CPE) was measured at two days, and 2) chromium^{51} release in which suspensions of target cells were labeled with radioactive Cr^{51} and lymphocytes added to the suspensions. Following interaction at varying lengths of time ranging from 4 to 24 hours, the amount of release of Cr^{51} into the supernatant fluids was measured.

Results.

Cytologic studies

In preliminary experiments employing a morphologic end point, the results of lymphocyte target cell interactions are shown in Table X. It can be seen in individuals who lack a history of natural measles or who had not been previously immunized and were seronegative consistently gave a pattern of response which was significantly less than the degree of CPE shown by seropositive individuals who had acquired their antibody either through natural disease or through immunization. Similar results were obtained in several repeated experiments. However, because of a lack of sensitivity a second mode of testing was employed, namely, the chromium^{51} release method.

Cr^{51} release experiments.

This testing employs the release of Cr^{51} from target cells which have been labeled with chromium and which interact with specifically sensitized lymphocytes. It has the advantage of a greater sensitivity and ease of quantitation. Further, since the release begins at 4 to 6 hours after the lymphocyte target cell interaction instead of 2 days, it is more readily determined.

The first set of experiments were designed to measure the rate of spontaneous release of Cr^{51} from labeled target cells. The results of these experiments are shown in Table XI. It can be seen that the rate of spontaneous release of Cr^{51} from both control and infected cells employing either HeLa or WI38 cells was a slow process. A maximum of 25% spontaneous release was noted by 24 hours. In subsequent experiments, 4 to 6 hours were selected as the endpoint for measurement of specific release when lymphocytes were added.

Effect of varying lymphocyte target cell ratios.

The next set of experiments were designed in order to determine the effect of different ratios of lymphocyte target cell and their release of Cr^{51} (Table XII). These studies employed lymphocytes from an individual previously shown to be reactive. It can be seen that below ratios of 100:1, it was difficult to measure release. Accordingly, in subsequent experiments, ratios of lymphocytes to target cells were maintained at 100:1 or higher.

Effect of measles immunity on Cr^{51} release.

Lymphocytes were obtained from individuals who had had natural measles previously or who had intercurrent measles at the time of the study. The results are shown in Table XIII. It can be seen that the net release of chromium from the individuals having had previous natural measles was about 5 or 6%. In contrast, two children with intercurrent measles at the time of study showed net chromium releases of 7 and 16%. These have been subsequently repeated in several experiments with a high degree of precision.

Conclusions.

The results of these studies indicate that an in vitro method of cell-mediated immunity to measles virus has been developed which is highly reproducible , sensitive and can be quantitated. In the limited studies that have been performed todate, it would appear that responsiveness can be detected in individuals having had natural measles or who have been immunized. There appears to be no correlation between the serologic reactivity and the cell-mediated reactivity from the limited number of studies that have been performed todate. It is anticipated over the coming year's support that these studies be continued and that they be extended to studies of more individuals. It seems possible that this method may provide a more sensitive and important biologic marker of immunity and can be provided by the study of serum antibody alone.

D. Other studies related to the basic proposal supported by the contract

Herpes simplex encephalitis: virologic and immunologic study of a patient treated with an interferon inducer.

A biopsy proven case of herpes simplex encephalitis was studied in a 4 1/2 month old male infant in whom transmission apparently occurred from an infected sibling who had gingivostomatitis.

Virological studies.

Herpesvirus was isolated from both vesicular fluid of a lesion on the foot and brain biopsy material. The first passage herpesvirus isolate, originally cultured in WI38 from the brain biopsy suspension, had a titer of $10^{5.5}$ $TCID_{50}$ per ml in WI38 cell culture tubes.

Results obtained when the virus isolates from the patient were cross-neutralized against type-specific HSV rabbit antiserum are given in Table XIV. Using established criteria, a pN difference between type 1 and type 2 of $\leq +0.05$ was considered to be a type 2 response and a pN difference of $\geq +0.5$ was considered to be a type 1 response. The data show that both the brain and vesicular fluid isolates were type 1 herpesviruses.

Serological Studies.

Herpesvirus complement-fixing and neutralizing antibody titers were determined in sera from both patient D.R. and his sibling A.R. (Table XV). It can be seen that the patient's CF titers ranged from 1:2 in his initial specimen to 1:512 three months later. The CF antibody titers of his sibling, A.R. revealed initial levels of 1:32, nearly four weeks after clinical onset of gingivostomatitis, and 1:512 two months later.

Quantal microneutralization studies over a three month period on D.R. revealed a very small amount of neutralizing antibody in the acute specimen with a greater than four-fold rise in HSV neutralizing antibody titer to types 1 and 2, but greater for type 1 (Table XIV). The pN difference in all three specimens indicated a type 1 response. Initial serum specimen on A.R. taken nearly one month after the onset of gingivostomatitis, revealed a moderate amount of type 1 neutralizing antibody.

A serum specimen two months later, demonstrated a greater than four-fold rise to both types of HSV, and the pN difference in both specimens indicated a type 1 response.

In both D.R. and A.R. a consistent picture of maximal pN difference is shown in the convalescent sera samples, indicating a large amount of specific type 1 antibody (relative to type 2) 3-4 weeks following infection. With time, however, some increase in the presumably cross-reacting type 2 neutralizing antibody was seen; nevertheless, antibody specificity could still be determined by pN differences and the continued rise in type 1 antibody.

In·Cn Dosage Schedule and Interferon Assays

Figure 2 depicts the dosage schedule of the In·Cn administered intravenously (I.V.) and the interferon assays which were determined prior to and during the administration of the drug. Specimens for interferon assay were obtained usually within 24 hours of the last dosage of drug with the exception of the first two days when specimens were obtained at more frequent intervals. Interferon was detectable in CSF and serum but not in urine prior to the administration of In·Cn. In general, no impressive serum or CSF interferon elevation was seen above that measured in baseline levels. Continued production of interferon, lasting through the 12th day appeared to coincide with the duration of treatment. The interfering principle was characterized further as interferon by having the following properties: (1) cell-bound antiviral effect, (2) activity against antigenically unrelated viruses (Sindbis and vesicular stomatitis viruses) (3) failure of neutralization of antiviral activity by antiserum against herpes simplex virus, and (4) the demonstration of cell species-specificity of antiviral activity.

The present case represents the first known description of treatment in a human with the interferon inducer In·Cn, and the first documented interferon studies of HSV encephalitis in man. It is difficult to assess the therapeutic benefit which was obtained in this patient following the use of the drug. Further, since interferon levels were elevated prior to the administration of the drug (presumably related to the acute infection) it was not possible to establish that interferon induction by In·Cn had occurred. In recent studies, similar doses of In·Cn gave rise to about 20 - 100 units/ml of serum interferon within 24 hours but little or no interferon response to repeated daily doses. The intranasal administration of In·Cn in

experimental rhinovirus infections in humans has been shown to decrease the frequency and severity of illness and to reduce virus shedding. In the present case, such small rises of interferon upon stimulation by In·Cn would have been masked by the high levels of virus-induced interferon which persisted until the 8th day. Also, the prior presence of virus-induced interferon would be expected to result in a much reduced interferon response to In·Cn (hyporesponsiveness). Nevertheless, it is possible that the continued production of low levels of interferon observed toward the end of treatment, represented, at least in part, an effect of In·Cn.

The presence of high levels of interferon in the serum and CSF before onset of treatment indicates that severe HSV infection is, indeed, a potent stimulus of interferon in man as has been reported for other viral infections including those of the nervous system. Additional studies in man are needed to establish further the capability of HSV to stimulate interferon in serum and CSF and to determine the role of interferon in recovery from infection with this virus.

IV. Publications

1. Bellanti, J.A., Klutinis, B., Sanga, R.L.: Antibody Responses in Serum and Nasal Secretions of Children Immunized with Rubella Vaccines. (in preparation).

2. Bellanti, J.A., Cantz, B.E., and Schlegel, R.J.: Accelerated Decay of Glucose-6-Phosphate Dehydrogenase Activity in Chronic Granulomatous Disease. Ped. Res. (in press) 1970.

3. Bellanti, J.A., Yang, M., Bartelloni, P.J., Beisel, W., and Crozier, D.: Studies of Leukocyte Function during Experimental Sandfly Fever Virus Infection in the Human (in preparation).

4. Bellanti, J.A., Cantz, B.E., Yang, M., von Thadden, H., and Schlegel, R.J.: Leukocyte Maturation: G-6-PD Activity and NBT Dye Reduction. Society for Pediatric Research, Atlantic City, N.J., 1970.

5. Bellanti, J.A., Catalano, L.W.,Jr., and Chambers, R.W.: Herpes Simplex Encephalitis: Virologic and Serologic Study of a Patient Treated with an Interferon Inducer. J. Pediat. (submitted for publication)

6. Bellanti, J.A., Artenstein, M.S., Brandt, B.L., Klutinis, B.S., and Buescher, E.L.: Immunoglobulin Responses in Serum and Nasal Secretions after Natural Adenovirus Infections. J. Immun. 103:891, 1969.

7. Bellanti, J.A., Sanga, R.L., Tina, L.U., Klutinis, B. and Ward, P.A.: Factors leading to the local production of respiratory antibody: The problems associated with immunologic imbalance, "Secretory Immunologic System" Workshop, Vero Beach, Fla., 1970 (in press).

8. Bellanti, J.A.: Studies of local and serum antibody responses to replicating agents. "Secretory Immunologic System" Workshop, Vero Beach, Fla. 1970 (in press).

TABLE I

Development of Serum HI Antibody Following Immunization with Rubella Virus Vaccine

Subject	Reciprocal Titer on Indicated Day							
	0	14	28	42	56	70	84	98
1	< 4	< 4	256	256	512	512	512	256
2	< 4	32	256	256	512	512	512	NT *
3	< 4	4	8	4	4	4	128	128
4	< 4	< 4	128	32	NT	NT	NT	NT
5	< 4	< 4	16	8	8	4	4	3
6	< 4	< 4	4	NT	NT	NT	NT	NT
7	< 4	< 4	512	1024	2048	NT	NT	NT
8	< 4	< 4	128	256	256	128	NT	NT
9	< 4	4	512	512	512	NT	NT	NT
10	< 4	< 4	256	128	256	256	256	128

* NT = Not Tested

Development of Neutralizing Antibody in Nasal Secretions Following Immunization with Rubella Virus Vaccine

Subject	Reciprocal Titer on Indicated Day							
	0	14	28	42	56	70	84	98
1	< 1	< 1	< 1	< 1	8	8	2	2
2	< 1	< 1	4	8	1	4	1	NT *
3	< 1	1	≥ 8	4	2	1	< 1	< 1
4	< 1	4	8	< 1	NT	NT	NT	NT
5	< 1	4	16	4	8	16	≥ 16	≥ 16
6	< 1	< 1	≥ 16	NT	NT	NT	NT	NT
8	< 1	< 1	8	1	4	NT	NT	NT
9	< 1	NT	2	8	≥ 16	NT	NT	NT
10	NT	4	4	4	1	2	4	4

* NT = Not Tested

Table III

Laboratory findings in patients with CGD and normal subjects

				Leukocyte	
Subjects	Age (years)	Bactericidal 1/ Activity (%)	NBT 2/	G-6-PD Activity 3/	6-PGD Activity 3/
Patients					
S.E.	7	70	0.022	10	26
J.M.	2-1/2	20	0.010	26	43
B.P.	4	60	0.015	11	49
Controls					
K.A.	7	90-95	0.320	70	24
K.B.	3		0.125	100	42
K.C.	13		0.190	161	50
J.F.	40		0.250	100	36

1/ Bactericidal activity is expressed as the per cent reduction in viable bacteria after 120 minutes incubation with leukocytes.

2/ NBT results are expressed as ΔOD at 515 mμ/2.5×10^{6} leukocytes/15 minutes at 37°C.

3/ Enzyme activity is expressed as μmole/min/gm protein.

Table IV

Effect of storage at 4°C on leukocyte and erythrocyte G-6-PD activities

Subjects	Age (years)	Leukocyte 1/ %			Erythrocyte 1/ %	
		Hours			Hours	
		0	4	18	0	18
Patients						
S.E.	7	100	42	0	100	58
S.E. 2/		100	74	0	-	-
B.P.	4	100	45	0	100	66
B.P. 2/		100	28	0	-	-
Controls						
Adult	30	100	95	58	100	93
Adult 2/	40	100	52	12	100	80
P.M.	7	100	86	71	100	100

1/ Enzyme activities for leukocytes or erythrocytes are expressed as % of activity measured at 0 time.

2/ Enzyme activities on cell homogenates prepared immediately without prior freezing.

Table V

Effect of stabilizing agents on leukocyte and erythrocyte G-6-PD activities

Subjects	Treatment	Leukocytes 1/ (%) Hours 0	4	18	Erythrocytes 1/ (%) Hours 0	18
Patients						
S.E.	0	100	42	0	100	65
	NADP	100	50	0	100	90
	2-ME	100	100	32	-	-
	2-ME + NADP	100	83	40	100	91
B.P.	0	100	45	0	100	66
	NADP	100	53	0	100	100
	2-ME	100	80	36	-	-
	2-ME + NADP	100	56	41	100	100
Controls						
P.M.	0	100	86	71	100	100
	2-ME + NADP	100	100	82	100	100
Adults (3) 2/	0	100	95	58	100	93
	NADP	100	100	80	100	94
	2-ME	100	78	88	-	-
	2-ME + NADP	100	100	88	100	96

1/ Enzyme activities for leukocytes or erythrocytes are expressed as % of activity measured at 0 time.

2/ Average values for 3 adults.

Table VI

Heat stability of leukocyte and erythrocyte G-6-PD activities at 38°C

Subject	Protein conc. (ug/ml)	Leukocyte 1/ (%)				Hemoglobin conc. (gm/1000 ml)	Erythrocyte 1/ (%)			
		Minutes					Minutes			
		0	10	20	30		0	10	20	40
Patients										
S.E.	880	100	40	17	0	11	100	100	87	67
B.P.	500	100	30	10	2	10.1	100	91	82	68
Control										
M.M.	600	100	60	51	34	12	100	88	81	78
	132	100	47	25	12					

1/ Enzyme activities for leukocytes or erythrocytes are expressed as % of activity measured at 0 time.

TABLE VII

Quantitative NBT Dye Reduction During Infection with Sandfly Fever Virus

Subject No.	NBT[a] values on indicated day						
	0	1	3	4	7	19	27
Controls							
7	0.075	0.125	0.060	0.054	0.108	0.121	NT
8	0.077	0.061	0.070	0.060	0.098	0.123	NT
16	0.172	0.243	0.221	NT	0.207	NT	0.219
Patients							
1	0.145	0.148	0.136	0.070	0.031	0.115	NT
2	0.098	0.057	0.052	0.023	0.105	0.128	NT
3	0.163	0.082	0.141	0.084	0.098	0.176	NT
4	0.106	0.140	0.071	0.109	0.061	0.134	NT
5	0.096	0.082	0.089	0.060	0.054	0.150	NT
6	0.146	0.151	0.122	0.105	0.045	0.172	NT
9	0.115	0.153	0.052	NT	0.081	NT	0.130
10	0.179	0.223	0.228	NT	0.028	NT	0.212
11	0.275	0.243	0.221	NT	0.207	NT	0.219

Nitroblue tetrazolium (NBT) expressed as ΔO.D. at 515 mμ/2.5 x 10^6 leukocytes/15 min at 37°C.

TABLE VIII

Leukocyte G-6-PD Activity During Infection with Sandfly Fever Virus

	G-6-PD Activity[a] on indicated day							
	0	1	3	4	7	19	25	32
Controls								
7	93	132	88	111	108	80	NT	NT
8	112	116	146	143	117	64	NT	NT
16	137	NT	154	NT	164	NT	155	152
Patients								
1	103	153	148	98	21	31	NT	NT
2	104	114	127	57	31	38	NT	NT
3	108	145	123	75	17	152	NT	NT
4	91	112	74	121	20	11	NT	NT
5	91	104	131	105	6	7	NT	NT
6	107	133	117	130	6	58	NT	NT
9	114	NT	109	NT	10	NT	99	128
10	119	NT	115	NT	4	NT	112	62
11	157	NT	155	NT	158	NT	66	122

G-6-PD activity expressed as mμ M NADPH/min/mg of protein.

TABLE IX

Leukocyte 6-PGD Activity During Infection With Sandfly Fever Virus

	6-PGD Activity[a] on indicated day					
Subject	0	1	3	4	7	19
Controls						
7	32	26	47	40	37	53
8	34	28	46	26	25	68
Patients						
1	35	28	47	31	30	50
2	37	27	38	28	31	47
3	28	23	38	26	25	50
4	33	21	42	32	28	57
5	34	38	36	27	19	47
6	36	46	41	30	34	58

6-PGD activity expressed as mμ M NADPH/min/mg of protein.

TABLE X

Results of Lymphocyte-Target Cell Interaction as Determined by Morphologic Means

	Subject	AGE	Measles History	Measles N titer	Degree of CPE
Sero-negative	H.W.	25	no measles; no immunization	<1:4	+
	B.S.	5 1/2 yrs	no measles; no immunization	<1:4	+
	V.S.	4 1/2 yrs	no measles; no immunization	<1:4	+
Sero-positive	J.B.	35	natural measles 20 years ago	1:8	4+
	A.S.	7	live measles vaccine	1:128	3+
	G.S.	8	live measles vaccine	1:32	4+

+ - 25% of cell sheet destroyed
+2 - 50% of cell sheet destroyed
+3 - 75% of cell sheet destroyed
+4 - 100% of cell sheet destroyed

TABLE XI

Rate of Spontaneous Release of Cr^{51}

Exp.	Target Tissue	% release of Cr^{51} at indicated hours					
		0	2	4	6	18	24
#1	Control	3	7	8	9	17	16
	Infected	4	10	11	15	23	25
#2	Control	5	9	11	15	21	23
	Infected	4	8	8	12	17	21

TABLE XI

Rate of Spontaneous Release of Cr^{51}

Exp.	Target Tissue	% release of Cr^{51} at indicated hours 0	2	4	6	18	24
#1	Control	3	7	8	9	17	16
	Infected	4	10	11	15	23	25
#2	Control	5	9	11	15	21	23
	Infected	4	8	8	12	17	21

TABLE XII

Effect of Different Lymphocyte:Target (L:T) Cell Ratios on Rate of Cr^{51} Release

Time (hrs)	L:T ratio	% Release Control	% Release Infected	Not Released
6 hrs	200:1	5	4	-1
	100:1	3	3	0
	50:1	2	2	0
17 hrs	200:1	7	11	4
	100:1	5	5	0
	50:1	3	3	0

TABLE XIII

Effect of Measles Immunity on Cr^{51} Release

Subject	Measles hx	Measles N titer	Time (m)	% Release		
				Control	Infected	Net Cr^{51} Release
J.B.	Measles 30 years previous	1:8	0	0	1	1
			4	2	2	0
			8	3	2	-1
			19	0	6	6
B.K.	Measles 20 years previous	1:32	6	2	4	2
			19	1	6	5
A.Mc.	Intercurrent measles	1:4	6	9	10	1
			19	9	15	7
D.K.	Intercurrent measles	1:8	6	2	16	14
			19	2	18	16

TABLE XIV

MICRONEUTRALIZATION STUDIES OF HERPES VIRUS ISOLATES FROM CASE REPORT USING TYPE-SPECIFIC RABBIT ANTISERUM

		MICRONEUTRALIZATION TEST WITH TYPE-SPECIFIC ANTISERUM						
		Neutralizing Antibody Titer		Neutralizing Potency Titer (pN)		pN Difference		
Virus	Microtiter */ml	Type 1	Type 2	Type 1	Type 2	Type 1	Type 2	Herpes Virus Type
Brain Isolate	$10^{7.11}$	1:380	1:10	3.077	1.497	+1.580		1
Vesicle Isolate	$10^{7.60}$	1:211	1:12	2.923	1.692	+1.241		1
Control type 1 (VR_3)	$10^{7.00}$	1:519	1:30	3.038	2.094	+0.944		1
Control type 2 (MS)	$10^{6.35}$	1:46	1:36	1.990	2.207	-0.217		2

---

* In $TCID_{50}$

TABLE XV

SEROLOGICAL STUDIES OF PATIENT AND SIBLING

			MICRONEUTRALIZATION TEST					
			Neutralizing Antibody Titer		Neutralizing Potency Titer (pN)		pN Difference	CF* Antibody Titer
Serum	Date	Type of Specimen†	Type 1	Type 2	Type 1	Type 2	Type 1 - Type 2	
tient D.R.	2-9-69	A	1:17	1:2	1.720	0.932	+0.788	1:2
tient D. R.	2-24-69	C	1:190	1:13	2.774	1.739	+1.035	1:64
tient D. R.	5-8-69	PC	1:452	1:87	3.043	2.328	+0.715	1:512
bling A.R.	2-25-69	C	1:129	1:4	2.605	1.234	+1.371	1:32
bling A. R.	4-29-69	PC	1:761	1:95	3.270	2.511	+0.759	1:512

A - acute, C - convalescent, PC - postconvalescent

Complement fixation test

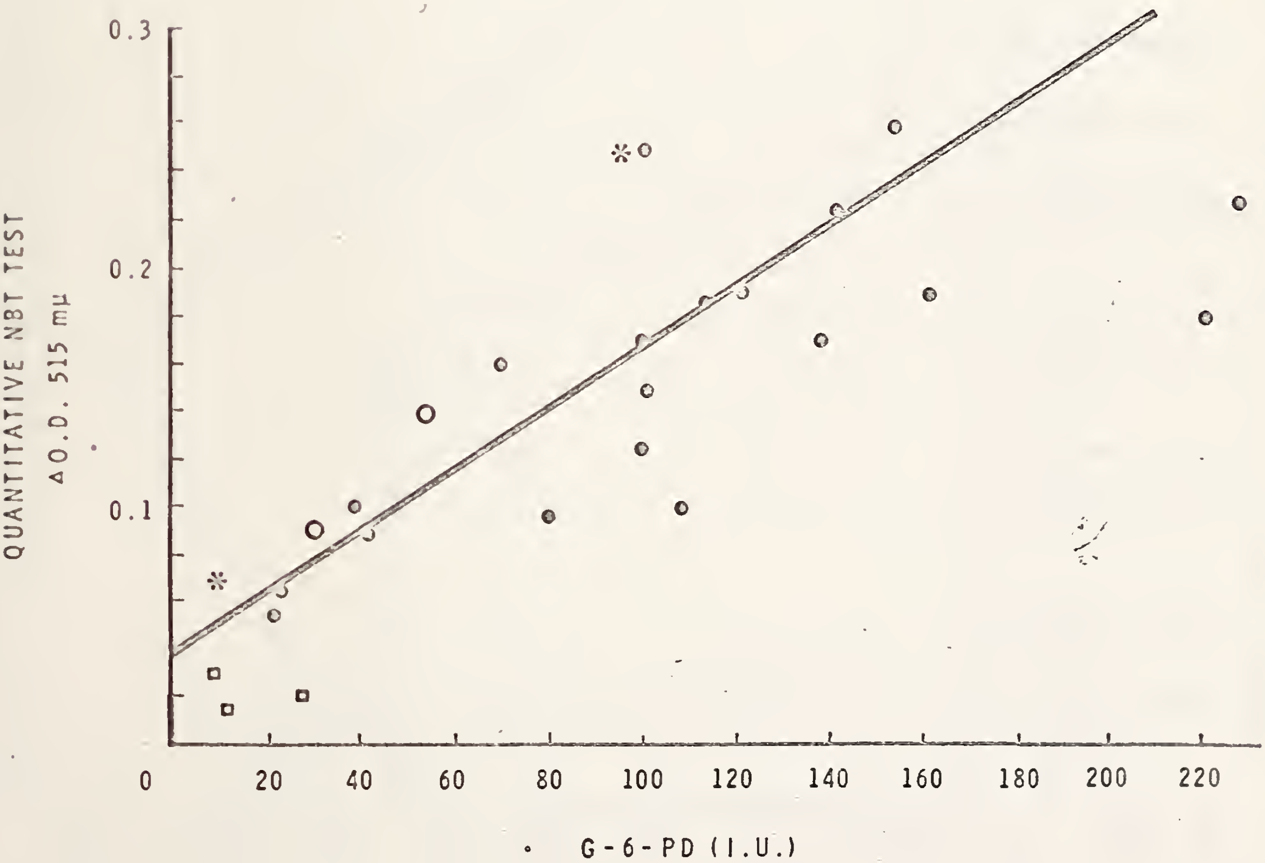
Laboratory Data of Patient, D.R., following In·Cn Treatment

Hospital Day	In·Cn Dose*	WBC (/mm^3)	Platelets (/mm^3)	Reticulocytes (%)	Prothrombin Time (%)	Transaminase		LDH	BUN mg%	Alkaline Phosphate	Total Bilirubin mg%	CSF Findings
						SGOT	SGPT					
1	Baseline +	8300	310,000	0.9	100	100	32	1095	8	1.7	0.8	RBC-12,250/mm^3 WBC - 4/mm^3 (3 lymphs, 1poly) Protein - 80 mg% Glucose - 64 mg%
2	+	10,300	155,000	1.7		152	42					
3	+	9,000	163,000	0.5		130	48		4	1.6	1.2	
4	+	7,900	165,000	0.2					5		2.0	
5	+					230		900	5		0.8	
6	+	5,800	179,000	1.4								
7	+	5,300	122,000	2.6	100	150	80	679				
8	+	4,900		1.6		150	48		5	1.7	0.9	RBC - 58/mm^3 WBC - 40/mm^3 (28 lymphs, 12 polys) Glucose - 66 mg%
9	+	6,200	292,000	2.0					9		0.8	
10	+	8,600	436,000	2.4					9		0.8	
11	+	6,400	365,000	2.2			40		16		0.4	
12	0	8,100	372,000	4.0	100	107	104	4250	16	3.0	0.4	
13	0	6,300	197,000	4.5						3.0		
14	0	10,100	443,000	4.2								
15	0	7,800	167,000	4.7		108	152		13	3.1	0.8	RBC - 11/mm^3 WBC - 26/mm^3 Glucose - 54 mg% Protein - 115 mg%
17	0	8,500	372,000	4.4					10		0.4	

* See text for explanation of dosage regimen

Legend

Figure 1. Correlation of leukocyte quantitative nitroblue tetrazolium test and glucose-6-phosphate dehydrogenase activity in humans of differing ages. Correlation coefficient is 0.86, $p < .01$. Stars (*) refer to Negroes having the A- variant, open circles (o) to people of Mediterranean origin, squares (□) to patients with CGD and closed circles (o) to normals.



INTERFERON IN SERUM AND CSF OF 4 1/2 MONTH OLD ♂ WITH HERPES SIMPLEX ENCEPHALITIS TREATED WITH POLY I·POLY C

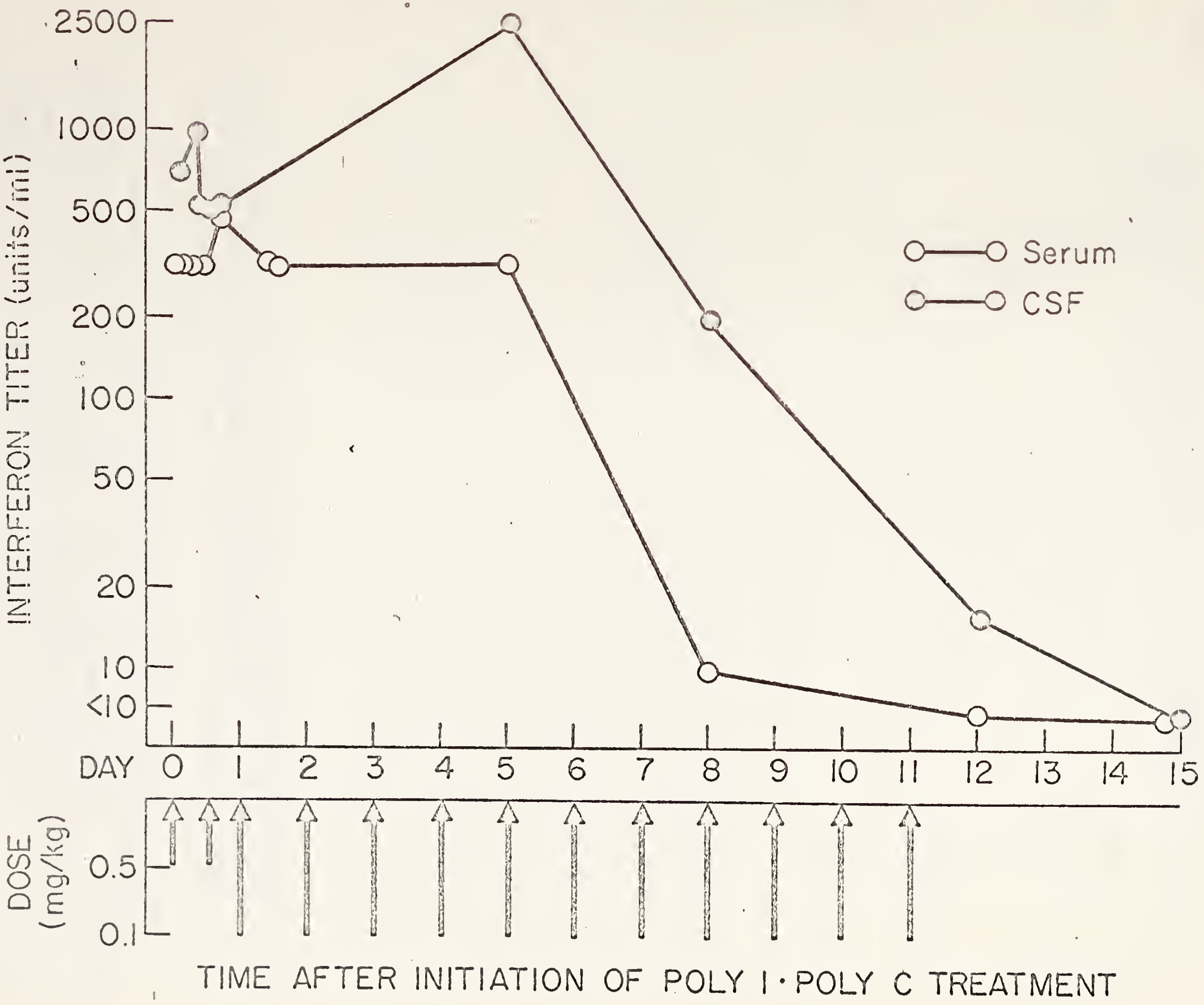


Figure 2. Interferon titers in CSF and serum in patient D.R. following intravenous polyinosinic·polycytidylic ribonucleic acid (In·Cn)

* Dosage was calculated on basis of 0.1 mg/kg/day administered as a single intravenous dose (1.0 mg/day) daily for 11 days with the exception of Day 1 when the drug was given as two equal doses.

** Unit of interferon is the reciprocal of the highest dilution of the sample which inhibits the single cycle yield of Sindbis virus by 0.5 $\log_{10}$ in AH-1 (human diploid cell line) cell cultures. Interferon assays were always performed on specimens drawn just prior to administration of a dose of In·Cn (for the first 11 days).

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13. ABSTRACT

This contract continues to be a study of the biologic significance of antiviral antibody in order to assess its role in immunity in the human. Control mechanisms and sequence data are being sought. The study of serum and local antibody responses has continued and has been performed in children immunized with live rubella vaccine.

The studies have been extended to include two other major areas concerned with antiviral immunity: 1) studies of biochemical and biologic events involved in phagocytosis, and 2) studies concerned with cell-mediated (delayed hypersensitivity) immunity to measles virus.

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14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
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ANNUAL PROGRESS REPORT

by

Frank M. Collins, Ph. D.

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Supported by

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SUMMARY

The present study was initiated to determine the feasibility of developing an oral mouse protection assay suitable for inactivated Salmonella vaccines. Preliminary studies disclosed that specific pathogen-free C57Bl mice were highly sensitive to oral infection with *Salmonella enteritidis* (oral LD_{50}= 5000 organisms). Starvation of the mice overnight, together with pretreatment with 10% bicarbonate solution increased susceptibility to oral challenge 2-5 fold. Quantitative organ counts were carried out on the liver, spleen, lung, mesenteric lymph nodes, stomach, small and large intestines and the cecum. Within 24 hrs. of infection with 10^5 *S. enteritidis*, the residual gut population had fallen to about 10% of the original challenge. By 48 hrs. the percentage of survivors was often as low as 1%. However, despite this rapid inactivation, considerable numbers of *S. enteritidis* reached the liver and spleen, and the mice subsequently underwent a severe systemic infection.

Mice were vaccinated with 1, 2 or 8 doses of 20ug (dry wt.) of a heat-killed saline suspension of *S. enteritidis*. They were orally challenged 8-14 days later with 10^5 *S. enteritidis*. The vaccinated mice showed little increased ability to limit or prevent the spread of the virulent challenge organism from the gut to the liver and spleen. Mice receiving the multiple doses of antigen were a little better protected than those receiving only the single dose. Good protection was observed against a strain of reduced virulence, however.

In conducting the research descrived in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee in the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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REPORT

The present report is necessarily short and incomplete since the project was only initiated on March 1, 1971. However, the preliminary findings have already indicated that the proposed study is entirely feasible. As a first step in the investigation, a detailed study of the quantitative changes occuring to a Salmonella population in the mouse gut and in the liver and spleen was undertaken.

1. Susceptibility of C57Bl and CD-1 mice to Salmonella challenge.

An earlier study (Collins 1970, Inf. and Immun. 1, 243-250) was made using specific pathogen-free CD-1 mice. This data indicated that serial organ enumeration of the developing liver and spleen populations permits the assessment of protection in mice orally infected with small infectious doses of S. enteritidis. Most earlier mouse protection studies employed relatively large oral challenge doses of virulent Salmonellae which result in the early death of 100% of normal animals. Prior drug treatment may increase host susceptibility considerably (Miller and Bohnhof 1963, J. Inf. Dis. 113, 59-66) but such a test system can hardly be considered a natural one. Protection was usually assesed in terms of an increased percentage of survivors amongst the vaccinated mice. However, the size of the challenge infection rendered much of this data open to the criticism that it was not biologically relevant to the natural disease (Collins 1971, J. Reticuloendothelial Soc. 9, in press). LD_{50} determinations with 3 strains of Salmonella indicated that the C57Bl mice are as much as 1000 times as susceptible to S. enteritidis and S. gallinarum infections, as the previously studied CD-1 strain of mice (Table 1). There was, however, no significant change in the LD_{50} for a mouse avirulent organism (S. pullorum). Two other strains of mice were tested for susceptibility to salmonellosis but both resembled the CD-1 strain in their overall sensitivities to these organisms (Table 1). The growth curves shown in Fig. 1 confirm that the C57Bl mice are exquisitely susceptible to oral S. enteritidis infection, since a few as 200 organisms given by mouth, induced a nonfatal systemic infection in at least 50% of the animals. The inbred mice were also more susceptible to S. gallinarum infections, as can be seen from the corresponding growth curves in Fig. 2.

2. Effect of prior starvation and bicarbonate treatment on susceptibility to Salmonellosis.

Mice were starved (but given water ad lib) for 24 hrs. before challenge. Half of the animals were fed 0.2 ml of 10% sodium bicarbonate solution and then both groups were challenged orally with 10^5 *S. enteritidis*, delivered directly into the stomach by gavage tube. Groups of normally fed mice, some also given bicarbonate pretreatment, were similarly challenged. Separate counts of the numbers of *S. enteritidis* in the stomach wall and contents, the small intestine wall and contents, the cecum and its contents and the large intestine and its contents were made after 1, 2, 6, 24 and 48 hrs. The gut wall preparatious were washed 4 times in saline to free them as much as possible from any residual intestinal contents. Homogenates of the washed walls and the corresponding contents were plated separately on desoxycholate citrate agar and the plates incubated at 37° for 48 hrs. Typical data taken from these experiments is presented in Tables 2-4. In addition, the liver, spleen, lungs and mesenteric lymph nodes were removed, homogenized and plated after 2, 4, 8, and 12 days. It can be seen that prior starvation and bicarbonate treatment increased the number of surviving *S. enteritidis* present in the stomach, small intestine and cecum over the first 24 hrs. Several thousand organisms were still present in the intestinal contents at 48 hrs. On the other hand, the normally fed animals contained very few intestinal Salmonellae after 24 hrs. (Table 4). Bicarbonate treatment of the normally fed animals also increased the persistance of the pathogen within the small intestine, but by 48 hrs. the numbers of surviving bacteria represented less than 1% of the initial challenge (Table 3). Despite the massive and rapid inactivation of the invading population within the intestines of the normal host, considerable numbers of *S. enteritidis* reached the liver and spleen where they were able to set up a progressive infection (Table 4). The lung cultures were always negative, at least until the terminal stages of the systemic infection. The typical growth curves representing the progress of the systemic infection resulting from challenge of C57Bl mice with decreasing doses of *S. enteritidis* (Fig.1) Showed striking similarities to the corresponding curves published earlier for the CD-1 mice (Collins, 1970). However, the size of the minimal infecting dose for the C57Bl mice was approximately 1000 times smaller. This material is being prepared for publication.

3. Vaccination of C57Bl mice with heat-killed vaccines.

S. enteritidis was grown overnight in a synthetic liquid medium at 37° C on a shaking machine (Collins and Mackaness, 1968, J. Immunol. 101, 830-845). The cells were removed by contrifugation at 4° C and washed once in sterile saline. The dense suspension was heated at 60° C for 30 minutes. Aliquots of the heated suspension were spread over the surface of typtose soy agar plates and incubated at 37° for 4 days as a check for sterility. No growth was observed. The heated cells were lyophilized without further washing. The dry powder was stored at room temperature *in vacuo.* The cells were homogenized briefly in saline at a concentration of 1 mg (dry wt.) per ml. and groups of mice were immunized subcutaneously, intraperitoneally, intravenously, intramuscularly or orally with 20 ug. of cells. Some mice received only 1 injection while others received 2 weekly injections. One group of mice received 8 doses of vaccine given by various routes at twice weekly intervals. All the mice were challenged orally 14 days after the final injection with 1-5 LD_{50} S. enteritidis. The vaccinated mice produced "O" agglutinating titers of from 1/10 to 1/40 with a maximum of 1/160 in the multiply vaccinated mice. The growth curves for the S. enteritidis challenges indicated that the heat-killed vaccine afforded the host very little real protection either in terms of preventing the pathogen from leaving the intestine and invading the tissues, or by the initiation of an accelerated immune response to the systemic infection (Fig. 3). However, mice given 8 doses of vaccine showed a somewhat slower rate of growth by the challenge organism in the livers and spleen. At the dose levels tested, killed vaccine given by the oral route afforded the host no protection what-so-ever.

In discussing the results achieved in this project so far, it should be pointed out that the work has not yet progressed very far, so that any conclusions must be tentative. It is clear that the use of serial counts of the numbers of Salmonellae in the various organ homogenates permits the experimentor to assess the affect of a given vaccine on the level of host resistance to oral infection, by at least 2 separate parameters. The first is the spread of the pathogen from the gut to the draining lymph nodes, liver and spleen, and the second is a comparison of the rate and extent of growth *in vivo* in the vaccinated and control mice. Death or survival of the challenged animals is not critical in this system. The host response to an unadjuvanted heat-killed vaccine is not sufficient to prevent systemic invasion or the initiation of clinical disease, even when a modest oral challenge is used. However, the poor levels of protection observed in the present study may be in part due to the use of a very highly virulent challenge organism. When the more attenuated Se 795 strain of S. enteritidis was used, a better level of protection was apparently achieved by the heat-killed Salmonella vaccine (Fig. 4). These experimental results will obviously require confirmation and amplification but at present, the data obtained is consistent with the state of affairs known to occur in human volunteers vaccinated with the TAB vaccine (Hornick _et al_ 1967, Med. Clin. N.A. _51_, 617-623).

Experiments using adjuvanted vaccines are now in progress and the use of water-in-oil emulsions would appear to offer the mouse improved protection against an oral challenge, compared with that afforded by the saline suspended, inactivated vaccines. These studies are, therefore, being actively pursued

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5. Hornick R.B., Woodward T.E., McCrumb F.R., Snyder M.J., Dawkins A.T., Bulkeley J.T., de la Macorra F., Corozza F.A. 1967. Typhoid fever vaccine - yes or no? Med. Clin. North Amer. 51, 617-623.

TABLE 1.

LD_{50}* determinations for orally and intravenously challenged mice.

Mice	Challenge route	*S. enteritidis* 5694	*S. gallinarum* 9240	*S. pullorum* 223
CD-1	I.V.	3×10^3	5×10^6	$> 10^8$
	B.M.	2×10^6	$> 10^8$	$> 10^8$
CF-1	I.V.	5×10^3	1×10^7	$> 10^8$
	B.M.	6×10^6	-	-
A/J	I.V.	4×10^2	2×10^5	-
	B.M.	5×10^5	-	-
C57Bl	I.V.	5 - 10	2×10^4	$> 10^8$
	B.M.	5×10^3	$7 - 10 \times 10^6$	$> 10^8$

* Determined by method of Reed and Muench (1938) after 28 days.

TABLE 2.

Logarithm of the numbers of viable S. enteritidis in the organs of C57Bl mice which had been starved overnight and given bicarbonate solution immediately before oral challenge with 3 x 10^5 bacteria.

	Time				
Organ	1 hr	2 hr	6 hr	24 hr	48 hr
Blood	< 2.0*	< 2.0	< 2.0	< 2.0	< 2.0
Spleen	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7
Liver	< 2.0	< 2.0	< 2.0	< 2.0	2.05 ± .40
Mesenteric nodes	< 2.0	1.92	< 2.0	< 2.0	3.51 ± .59
Stomach	4.20 ± .45**	3.60 ± .44	2.88 ± .45	< 2.0	< 2.0
Stomach contents	4.75 ± .68	4.22 ± .52	3.88 ± .45	< 2.0	< 2.0
Small intestine	4.9 ± .75	3.32 ± .89	2.48 ± .40	3.85 ± .46	4.21 ± .21
Small intestine contents	5.3 ± .68	5.12 ± .68	4.30 ± .66	-	3.70 ± .55
Cecum	3.10 ± .53	3.39 ± .53	3.72 ± .65	4.21 ± .65	4.27 ± .68
Cecum contents	4.8 ± .75	5.00 ± .61	4.61 ± .51	3.78 ± .36	3.90 ± .38
Large intestine	< 2.0	2.84 ± .66	2.67 ± .51	3.78 ± .36	2.90 ± .38
Large intestine contents	2.9 ± 1.00	5.54 ± .52	4.59 ± .52	3.39 ± .35	3.15 ± .22
TOTAL	5.62	5.46	5.06	4.58	4.78
% of inoculum	140	97	38	12	20

* Limit for viable count method

** Average of four determinations ± S.D.

TABLE 3.

Logarithm of the numbers of viable *S. enteritidis* in the organs of normally fed C57Bl mice given bicarbonate solution immediately before oral challenge with 3.4×10^5 bacteria.

	Time			
Organ	2 hr	6 hr	24 hr	48 hr
Blood	< 2.0	< 2.0	< 2.0	< 2.0
Spleen	< 1.7	< 1.7	< 1.7	1.8
Liver	< 2.0	< 2.0	< 2.0	2.83 ± .50
Mesenteric nodes	< 2.0	< 2.0	< 2.0	2.40 ± .53
Stomach	2.48 ± .52	2.48 ± .75	1.72 ± .88	< 2.0
Stomach contents	4.07 ± .76	3.55 ± 1.28	< 2.0	< 2.0
Small intestine	2.48 ± .55	2.50 ± .40	2.94 ± .35	3.20 ± .45
Small intestine contents	4.75 ± .56	2.70 ± 1.10	< 2.0	< 2.0
Cecum	3.59 ± .65	2.65 ± .48	< 2.0	< 2.0
Cecum contents	5.38 ± .41	4.95 ± .42	< 2.0	< 2.0
Large intestine	3.35 ± .15	-	< 2.0	< 2.0
Large intestine contents	4.58 ± .18	4.57 ± .68	< 2.0	< 2.0
TOTAL	5.23	5.13	3.27	3.20
% of inoculum	106	39	0.6	0.5

TABLE 4.

Logarithm of the number of viable S. enteritidis in the organs of normally fed C57Bl mice challenged orally with 4 x 10^5 bacteria without prior bicarbonate treatment

	Time			
Organ	2 hrs	6 hrs	24 hrs	48 hrs
Blood	< 2.0	< 2.0	< 2.0	< 2.0
Spleen	< 1.7	< 1.7	< 1.7	2.08 ± .15
Liver	< 2.0	< 2.0	< 2.0	2.81 ± .25
Mesenteric node	< 2.0	< 2.0	< 2.0	2.10 ± .50
Stomach	< 2.0	< 2.0	< 2.0	< 2.0
Stomach contents	2.73 ± .66	< 2.0	< 2.0	< 2.0
Small intestine	2.43 ± .21	2.35 ± .35	2.00 ± 1.5	< 2.0
Small intestine contents	4.39 ± .77	< 2.0	< 2.0	< 2.0
Cecum	3.36 ± .29	2.13 ± .21	< 2.0	< 2.0
Cecum contents	4.67 ± .22	3.43 ± .42	< 2.0	2.63 ± .88
Large intestine	3.16 ± .26	< 2.0	< 2.0	< 2.0
Large intestine contents	4.63 ± .19	4.08 ± .50	2.60 ± .40	2.30 ± .25
TOTAL	5.08	4.17	2.7	2.80
% of inoculum	30	4	0.1	0.2

Legends for text figures

Fig. 1. Growth curves obrained in C57Bl mice orally inoculated with decreasing doses of virulent *S. enteritidis*. (Top) 300 viable organisms; (middle) 5×10^3 viable bacteria; (bottom) 5×10^5 viable organisms. L = liver. S = spleen. B = blood. MN = mesenteric lymph nodes. G = gut. The inocula are represented by the arrows.

Fig. 2. Growth curves for *S. gallinarum* following (left) intravenous and (right) oral challenge of (top) C57Bl and (bottom) CD-1 mice. The oral challenge inoculum was approximately 10^7 viable organisms in both groups of mice. S = spleen. L = liver. MN = mesenteric lymph node. G = gut. The histograms represent the footpad swelling at 3 hrs. (solid bars) and 24 hrs. (broken bars) following injection of 1 ug. Salmonella test antigen into the right hind footpad.

Fig. 3. Growth curves in C57Bl mice subjected to an oral challenge with *S. enteritidis*. Mice were pretreated with 1, 2, or 8 subcutaneous injections of 20 ug. heat-killed *S. enteritidis* vaccine. The challenge was made 14 days after the final vaccine injection. Deaths were observed on days 8-12.

Fig. 4. Effect of virulence on the growth of challenge inocula of *S. enteritidis* in C57Bl mice vaccinated with 2 doses of 20 ug. heat-killed *S. enteritidis* vaccine. *S. enteritidis* strain 5694 (oral LD_{50} = 5000 organisms) and strain Se 795 (oral LD_{50} of 5×10^6 organisms) were both given by the oral route 14 days following the second dose of vaccine. The inocula are represented by the arrows.

SUMMARY

The restorative effect of peritoneal macrophages on delayed hypersensitivity following ionizing radiation.

Volkman A. and Collins F.M.

Cellular Immunology. 1971 2, in the press.

Sublethal whole body irradiation in the guinea pig had little demonstrable effect on the development of delayed hypersensitivity (DTH) but caused a profound, through transient depression of dermal reactivity in previously sensitized animals. Macrophage rich peritoneal cell suspensions from non-sensitive donors when injected intradermally with eliciting antigen, resulted in the restoration of a significant degree of reactivity following radiation. Inocula of lymphocytes did not yield similar results. These findings, when taken with the persistence of low levels of reactivity following radiation and the ability to transfer reactivity with peritoneal exudate cells, warrant the conclusion that macrophages are necessary for the expression of cutaneous DTH. The spontaneously renewed activity which follows the depressed phase is thus more a reflection of the recovery of macrophage precursors from radiation injury, rather than the emergence of a new population of sensitized cells. The results substantiate the belief that the expression of DTH requires two cell populations, only one of which carries the property of specificity at the outset. The presence of the senitized population cannot always be excluded by a weak or absent skin test.

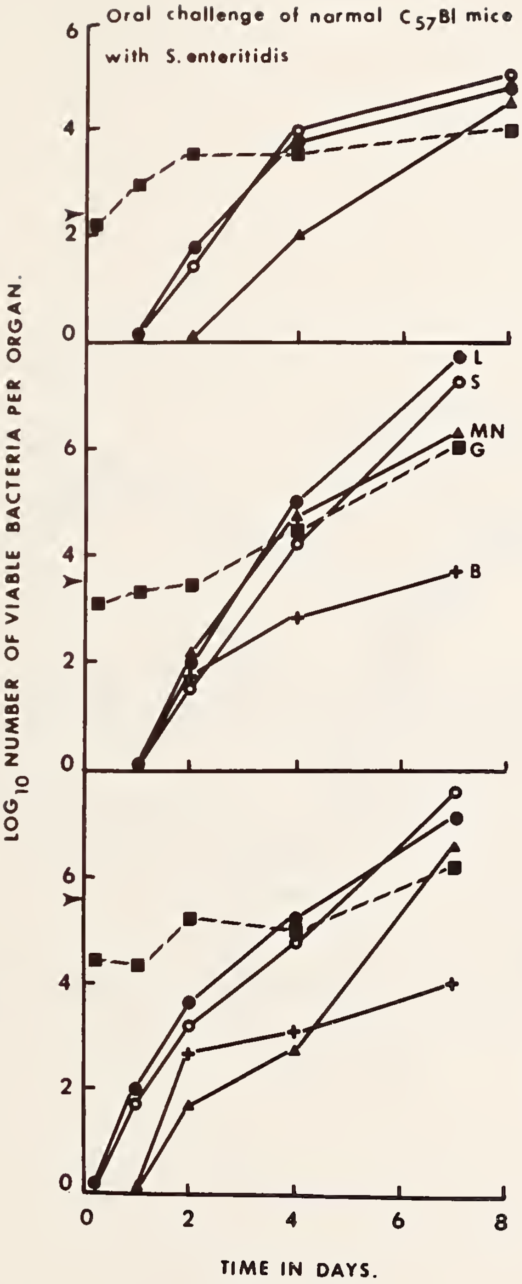
Oral challenge of normal C57Bl mice
with S. enteritidis
LOG10 NUMBER OF VIABLE BACTERIA PER ORGAN.
TIME IN DAYS.
L
S
MN
G
B
0
2
4
6
8

FIG. 1

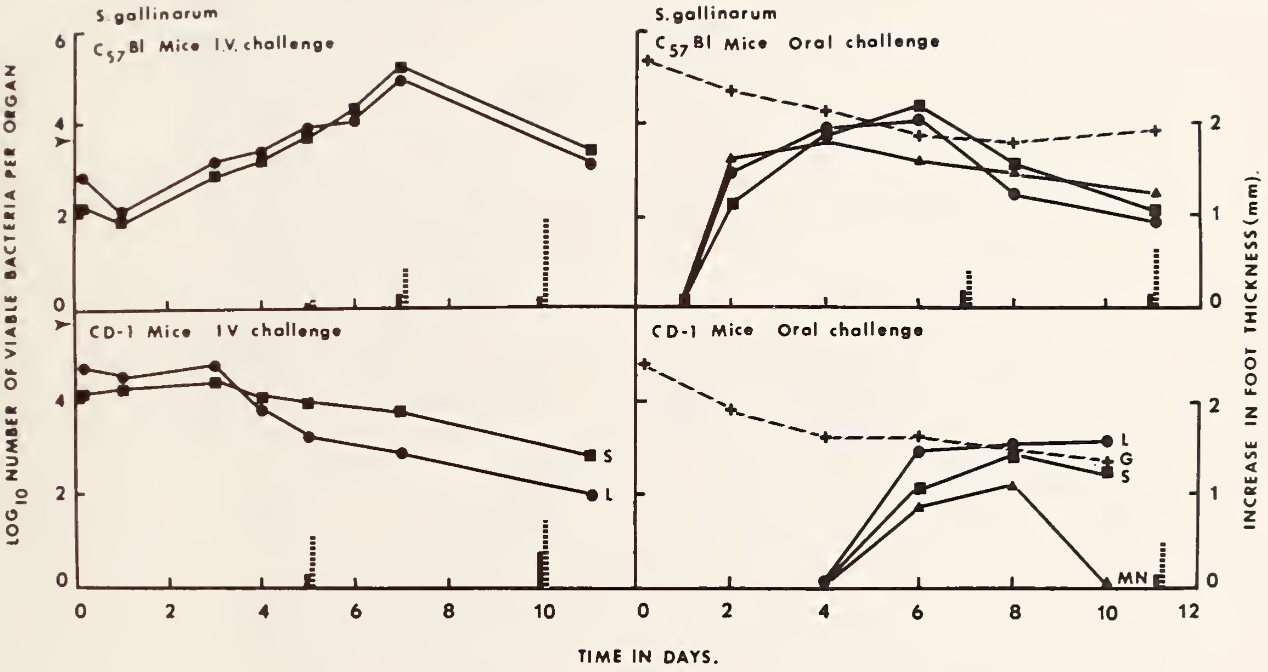
S.gallinarum
C57Bl Mice I.V. challenge
S.gallinarum
C57Bl Mice Oral challenge
CD-1 Mice IV challenge
CD-1 Mice Oral challenge
LOG10 NUMBER OF VIABLE BACTERIA PER ORGAN
INCREASE IN FOOT THICKNESS (mm).
TIME IN DAYS.
0
2
4
6
8
10
12
1
S
L
G
MN

FIG. 2

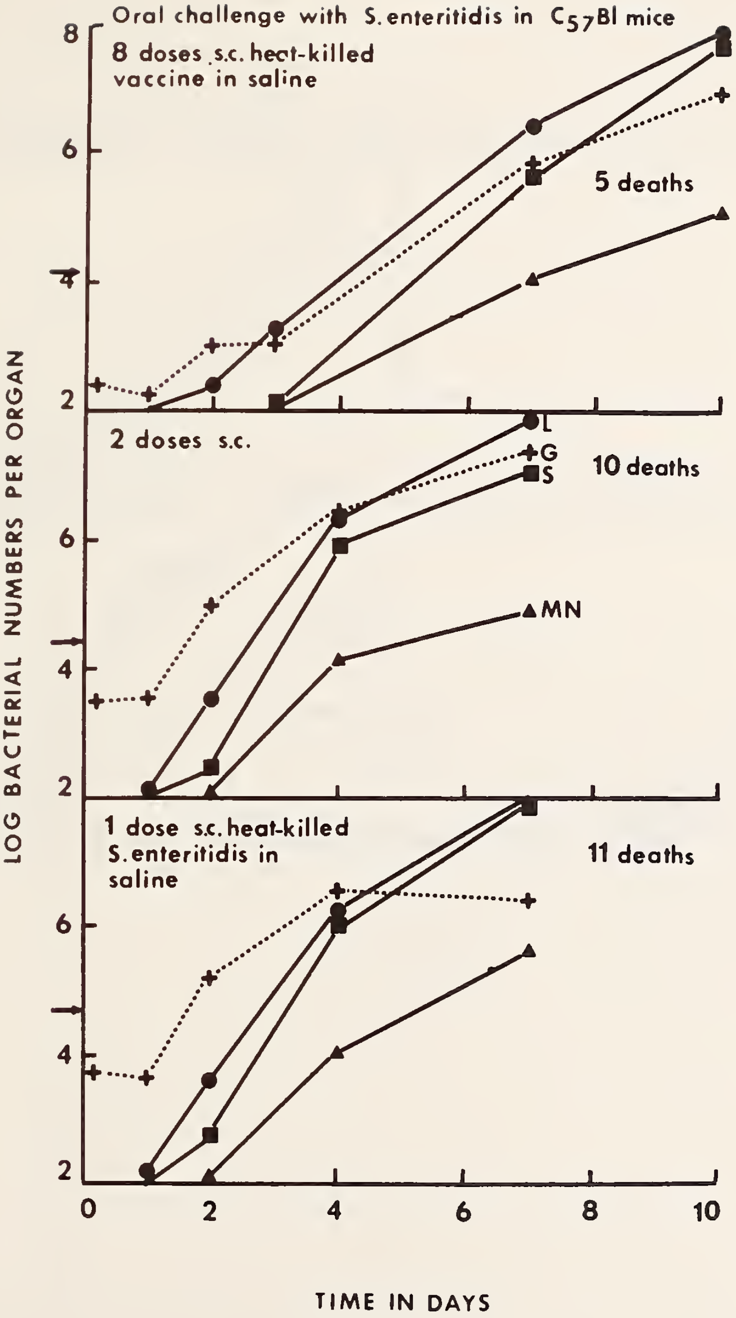
Oral challenge with S. enteritidis in C57Bl mice
8 doses s.c. heat-killed vaccine in saline
5 deaths
2 doses s.c.
L
G
S
MN
10 deaths
1 dose s.c. heat-killed S. enteritidis in saline
11 deaths
LOG BACTERIAL NUMBERS PER ORGAN
8
6
4
2
0
2
4
6
8
10
TIME IN DAYS

FIG. 3

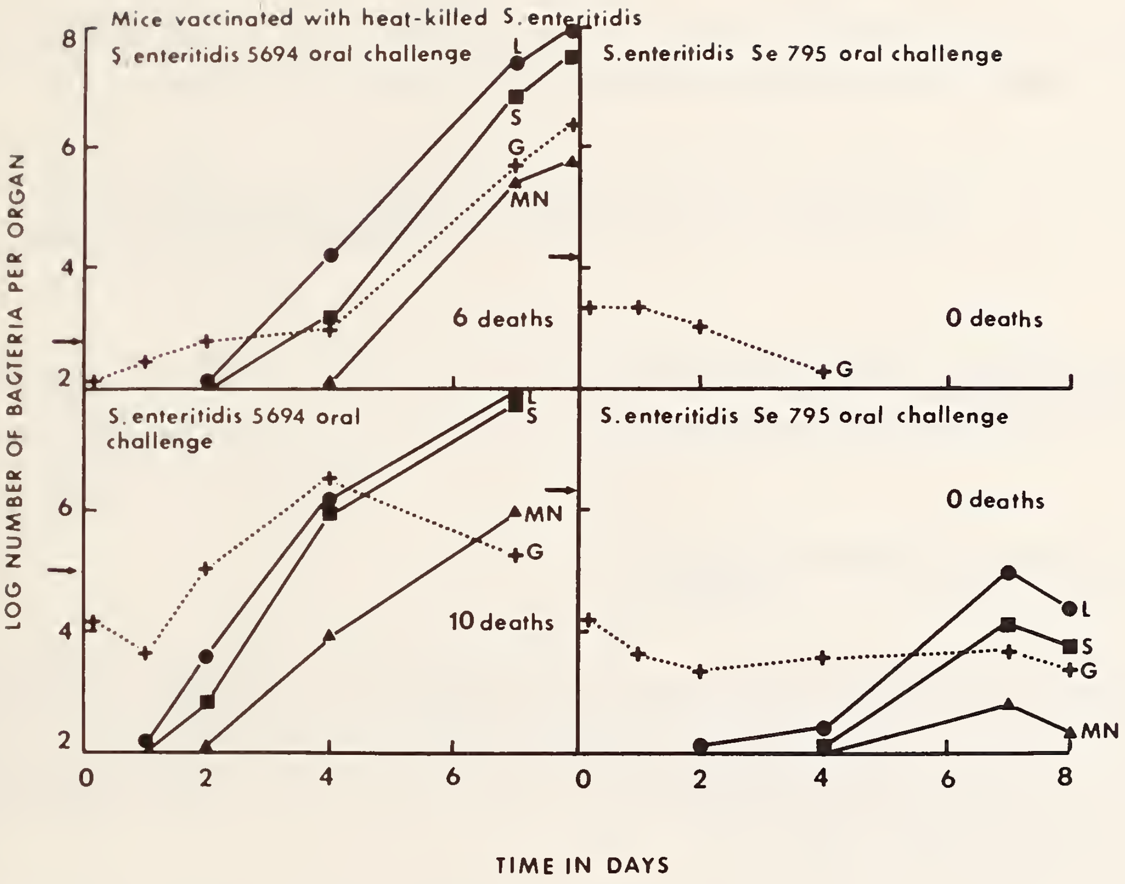
Mice vaccinated with heat-killed S. enteritidis
S. enteritidis 5694 oral challenge
S. enteritidis Se 795 oral challenge
6 deaths
0 deaths
S. enteritidis 5694 oral challenge
S. enteritidis Se 795 oral challenge
0 deaths
10 deaths
L
S
G
MN
LOG NUMBER OF BACTERIA PER ORGAN
TIME IN DAYS
8
6
4
2
0
2
4
6
8

FIG. 4

Security Classification

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY U.S. Army Medical Research and Development Command

13. ABSTRACT

The present study was initiated to determine the feasibility of developing an oral mouse protection assay for inactivated Salmonella vaccines. Preliminary studies disclosed that specific pathogen-free C57Bl mice were highly sensitive to oral infection with Salmonella enteritidis (oral LD_{50} = 5000 organisms). Starvation of the mice overnight, together with pretreatment with 10% bicarbonate solution increased susceptibility to oral challenge 2-5 fold. Quantitive organ counts were carried out on the liver, spleen, lung, mesenteric lymph nodes, stomach, small and large intestines and the cecum. Within 24 hrs. of infection with 10^5 S. enteritidis, the residual gut population has fallen to about 10% of the original challenge. By 48 hrs. the percentage of survivors was often as low as 1%. However, despite this rapid inactivation, considerable numbers of S. enteritidis reached the liver and spleen, and the mice subsequently underwent a severe systemic infection. Mice were vaccinated with 1, 2 or 8 doses of 20 ug (dry wt.) of a heat-killed saline suspension of S. enteritidis. They were orally challenged 8-14 days later with 10^5 S. enteritidis. The vaccinated mice showed little increased ability to limit or prevent the spread of the virlent challenge organism from the gut to the liver and spleen. Mice receiving the multiple doses of antigen were a little better protected than those receiving only the single dose. Good protection was observed against a strain of reduced virulence however.

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14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Typhoid Fever						
Vaccine						
Mouse						
Protection Test						
Oral Challenge						
Salmonella Enteritidis						
Delayed Hypersensitivity						
Cellular Immunity						
Attenuated Vaccine						
Inactivated Vaccine						
Adjuvant						
Acquired Antimicrobial Resistance						
Quantitative Organ Enumeration						
Secretory Antibody						

Security Classification

AD__________________

ROLE OF THE RETICULOENDOTHELIAL SYSTEM AND HUMORAL RECOGNITION FACTORS IN MALARIA INFECTION

ANNUAL PROGRESS REPORT

by

Nicholas R. Di Luzio, Ph.D.

(For the period 1 June to 31 May 1971)

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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Summary

The purpose of the research described in this report was to determine the role of the reticuloendothelial system (RES) in malaria infection. The primary hypothesis which was evaluated is that the macrophage is an essential element in host defense to malaria.

Using ^{51}Cr-labeled erythrocytes it was demonstrated that Plasmodium berghei infected erythrocytes were removed from blood at a significantly accelerated rate than normal non-infected erythrocytes. Neither stimulation, nor depression of the RES altered the phagocytic clearance of the labeled erythrocytes. However, certain RE stimulants such as diethylstilbestrol and melezitose, did significantly reduce parasitemia and mortality.

Utilization of the antimalarial agents quinacrine and chloroquine with the RE stimulant, glucan and RE depressant, methyl palmitate, demonstrated no functional synergism between the RES and anti-malaria drugs - an hypothesis which had been advanced by several investigators.

Since RES activity has been shown to be increased in a malaria infection and since stimulation of the RES is an event usually associated with endotoxin hypersensitivity, the susceptibility of malaria-infected mice to endotoxin was evaluated.

Malaria infected mice manifested a 40-fold increase in endotoxin susceptibility. While liver and spleen homogenates

from normal animals possessed a significant endotoxin-detoxification ability, liver homogenates prepared from malaria infected mice did not demonstrate endotoxin detoxification ability. The endotoxin detoxifying ability of splenic homogenates prepared from malaria infected donors was however normal.

These findings indicate that endotoxin hypersensitivity in malaria infected mice may be due to an impairment in hepatic endotoxin detoxification. Since endotoxin detoxification by liver has been predicated to be a function of Kupffer cells, the loss of endotoxin detoxifying ability in malaria infected animals adds a new dimension to macrophage functional alterations in malaria.

Foreword

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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1. Statement of Problem

Acquired immunity to malaria has been associated with an increased phagocytosis by macrophages and production of humoral antibodies. The aim of the present report was to study the relationship of the macrophage to a malaria infection in an effort to: (a) gain more insight into the basis of immunity to malaria, (b) project methods for enhancing specific acquired immunity, and (c) determine the effects of a malaria infection on the macrophage and the reticuloendothelial system (RES).

2. Background

Stimulation of the RES during the course of a malaria infection, as evaluated by vascular clearance of test colloids, has been demonstrated by numerous investigators (Cantrell and Elko, *J. Infect. Dis.* 116:429, 1966 and Sheagren et al., *J. Lab. Clin. Med.* 75:481, 1970). Histological examination of RE organs during a malaria infection has revealed a significant proliferation of the lymphoid macrophage tissue and an increased number of parasitized erythrocytes within the tissue macrophages. In addition to RE hyperfunction and hyperplasia, an increased concentration of circulating gamma globulin has been demonstrated in a malaria infection (Kuvin et al., *Am. J. Trop Med.* 11:429, 1962; Tobie et al., *J. Immunol.* 97:498, 1966 and Cohen et al. *Nature*, 192:733, 1961). Some of this gamma globulin has been suggested to be a specific antibody of the IgG class

(Cohen et al., *Nature*, 192:733, 1961) directed against the malaria parasite and protective to the host (Cohen et al., *Nature*, 192:733, 1961). These previous findings have led investigators to suggest that both phagocytosis of the malaria-infected erythrocyte by macrophages and the production of humoral antibodies is essential for the development of specific acquired immunity to malaria (Neva, *Ann. Int. Med.*, 114:837, 1961).

Processing of antigens by the macrophage has been suggested to result in the production of a macrophage immunogenic RNA (Fishman, *J. Exp. Med.* 114:837, 1961 and Fishman and Adler, *J. Exp. Med.* 117:595, 1963). Transfer of this RNA to an antibody producing cell with a concomitant production of a specific antibody has been postulated to be a function of the immunogenic RNA (Schwartz, et al., *Prog. Allergy*, 14:81, 1970).

Assuming that the macrophage processes malarial antigens in a similar manner to that of other particulate antigens (Frei et al. *Proc. Nat. Acad. Sci.* 53:20, 1965), then an enhancement of phagocytosis by the macrophage should possibly enhance the host response to the malaria parasite. Glucan, an RE stimulant, has indeed been demonstrated to produce an enhanced immune response (Wooles and Di Luzio, *Science* 142:1048, 1963). For this reason, glucan, in addition to diethylstilbestrol (DES) and melezitose (Di Luzio and Riggi, *J. Reticuloendothelial Soc.* 8:465, 1970) were used as RE stimulants.

Depression of RE activity by methyl palmitate has been demonstrated to result in a decreased immune response (Di Luzio and Wooles, Am. J. Physiol. 206:939, 1964), therefore, this agent was employed in addition to RE stimulants, to assay the importance of phagocytosis in a malaria infection.

Phagocytosis of the infected erythrocyte has been suggested to result in the production of antibodies to both the erythrocyte per se and the parasite (Kreier et al. Exp. Parasitol. 19:155, 1966). Such antibodies to the erythrocyte have been suggested to be a causative factor in the hemolytic anemia observed in a malaria infection. Indeed, the presence of anti-erythrocyte antibodies has led to the development of an autoimmune hypothesis to account for the malaria induced anemia (Zuckerman, Military Med. 131:1201, 1966). To test the validity of this concept, various investigators have employed anti-lymphocyte serum (ALS) as an immunosuppressive agent (Sheagren and Monaco, Science, 164:1423, 1969) with varying results.

Recent evidence in our laboratory has suggested ALS to have marked cytotoxic effects on macrophage cells (Pisano et al. Proc. Soc. Exp. Biol. Med. 132:517, 1969 and Pisano et al. Adv. Exp. Med. Biol. 15:237, 1971) implying that ALS may also be acting as an anti-macrophage cell serum. This may be a possible mode of action for the immunosuppressive activities of ALS since macrophage transplantation reversed ALS-induced

immunosuppression (Patterson et al., Proc. Soc. Exp. Biol. Med. 135:831, 1970). Therefore, studies were also initiated to evaluate ALS as an RE depressing agent and as an anti-malaria agent.

A functional RES has been suggested to be an essential element in the mechanism of action of various anti-malarial agents. In fact, a synergism between the RES and antimalarial agents has been reported by Bliznakov (Adv. Exp. Biol. Med. 15:315, 1971). To ascertain the presence of an RES-antimalarial drug synergism in a malaria infection, RE stimulants and depressants were employed in conjunction with chloroquine and quinacrine.

Stimulation of the RES in a malaria infection has been demonstrated by various investigators using either colloidal carbon (Cantrell and Elko, J. Infect. Dis. 116:429, 1966) or ^{125}I-microaggregated human serum albumin (Sheagren et al., J. Lab. Clin. Med. 75:481, 1970). Since stimulation of the RES is an event usually associated with endotoxin hypersensitivity (Suter et al., Proc. Soc. Exp. Biol. Med. 99:167, 1958 and Benacerraf et al., Proc. Soc. Exp. Biol. Med. 100:796, 1959), the susceptibility of malaria infected mice to endotoxin was evaluated. In addition, endotoxin detoxification has been predicated to be a function of the macrophage (Filkins, J. Reticuloendothelial Soc., 9:489, 1971). Since there is profound morphological and functional alterations of macrophages in a

malaria infection, the ability of liver and spleen, organs with large macrophage populations, to detoxify endotoxin was assayed.

Macrophages obtained from RE stimulated animals have been termed "activated" and have been demonstrated to have an enhanced bactericidal activity (Cooper, J. Pathol. Bacteriol. 89:665, 1965 and Lavelle, and Stan, Brit. J. Exp. Pathol. 50:475, 1969). Therefore, since a malaria infection induces an RE stimulation, macrophages from malaria infected animals were also assayed for their bactericidal activity.

3. Approach to the Problem

^{51}Cr-labeled erythrocytes were employed in an effort to compare the phagocytic uptake and tissue distribution of normal erythrocytes and malaria infected erythrocytes in normal, RE stimulated, and RE depressed animals. RE stimulants were glucan and melezitose, while methyl palmitate will be employed as an RE depressant. The effect of RE stimulation and depression in conjunction with the antimalarial drugs quinacrine and chloroquine on malarial parasitemia and mortality was evaluated to ascertain if a synergism exists between the RES and antimalarial agents. In addition, the immunosuppressant ALS was assayed for its ability to alter malaria infection. Endotoxin sensitivity in malaria infected mice and tissue detoxification of livers and spleens obtained from normal and malaria infected mice will be ascertained using previously described methods

(Trejo and Di Luzio, Proc. Soc. Exp. Biol. Med. 136:889, 1971). The bactericidal activity of macrophages from malaria infected mice will be evaluated by assaying the ability of peritoneal macrophages, derived from malaria infected mice, to kill S. aureus.

4. Research Summary

A. Influence of RE Stimulation and Depression of Intravascular Clearance and Tissue Distribution of Malaria-Induced Red Cells

In line with the primary hypothesis of this project, that the macrophage is an essential element in host-defense to malaria, we have investigated the rate of phagocytosis of P. berghei infected erythrocytes in RE altered mice.

In agreement with the findings of Kreier and Leste (Exp. Parasitology 21:78, 1967), it was found that erythrocytes obtained from malarial-infected animals were removed from the circulation faster than erythrocytes obtained from non-infected donors as t/2 were 15.8 hr for normal cells relative to 8.5 hr for malaria cells (Table 1). An exception to the general observation that malarial erythrocytes were removed more rapidly than normal erythrocytes occurred in the glucan-treated RE stimulated mice (Table 1).

The apparent normalization of intravascular removal rates, when considered in light of tissue distribution data (Table 2), implies that the RE stimulated mice were characterized by enhanced phagocytosis of the malaria-infected red cells. The

Table 1: Mean intravascular clearance of ^{51}Cr-labeled normal and malarial erythrocytes in RE stimulated and depressed mice.

Group	No.	Half-time, hours Malarial Erythrocytes	Normal Erythrocytes
Saline	6	8.5	15.8
Glucan	5	16.7	11.2
Tween 20, Control	6	7.0	19.5
Methyl Palmitate	6	10.1	18.5

Intravascular clearance rates determined following the injection of 1.0 x 10^8 red cells from normal or malaria-infected mice. Blood samples were obtained at 1, 3, 6, and 24 hours for determination of vascular clearance. The malaria-infected animals which served as red cell donors had parasitemias of 30%.

Table 2: Comparative organ uptake of ^{51}Cr-labeled normal or malaria-infected red cells in control and RE stimulated mice*

Group	Red Cells Injected	Blood Activity %ID/ml	Liver g	Liver %ID+	Lung g	Lung %ID	Spleen g	Spleen %ID
Saline	Normal	58.6	1.0	7.5	0.1	1.3	0.1	1.4
Glucan	Normal	54.9	1.4	7.5	0.2	1.4	0.3	1.7
Saline	Malaria	16.1	1.2	19.0	0.2	1.5	0.07	18.5
Glucan	Malaria	30.9	1.6	22.5	0.2	2.4	0.3	13.8

*Values are expressed as means and are derived from 5 to 6 mice per group. Data were obtained six hours following intravenous administration of labeled red cells.

+Percent injected dose per total organ.

normalization of the intravascular half-time, therefore, denoted the vascular clearance of the normal red cells in the injectate.

Liver uptake of ^{51}Cr-labeled red cells from malaria-infected mice was enhanced 3-fold over control mice and slightly enhanced over that value in glucan-stimulated mice. Lung uptake was essentially equivalent in all groups. Spleen uptake showed an enhancement of 13-fold in the saline group and was slightly decreased in glucan-treated mice.

Melezitose, a water-soluble trisaccharide which possesses RE stimulatory activity, was also evaluated for its ability to enhance phagocytosis of malarial-infected erythrocytes (Table 3). A significant decrease in the intravascular clearance rate of ^{51}Cr-labeled malarial erythrocytes was demonstrated in melezitose treated animals versus the saline control animals. A significant reduction in tissue localization of the labeled malaria erythrocytes was also observed in the liver, lung and spleen (Table 3).

In methyl palmitate-treated RE depressed mice, the vascular clearance of normal red cells was not modified (Table 4). The blood activity of ^{51}Cr-labeled malaria red cells was comparably decreased in the Tween and methyl palmitate groups. Organ uptake was comparable with the exception that the degree of enhancement in spleen uptake was decreased in the methyl palmitate-treated group. Similar findings were observed 24 hours following red cell administration and the values are not

Table 3: Effect of melezitose on clearance and tissue distribution of normal and malaria infected ^{51}Cr-labeled erythrocytes

Treatment	Cells	LIVER		LUNG		SPLEEN		T/2
		%ID/g	%ID/TO	%ID/g	%ID/TO	%ID/g	%ID/TO	Days
Melezitose+ (n=9)	Malaria	11.3*	17.6*	3.2*	0.6*	39.0*	8.8*	3.2*
Saline (n=9)	Malaria	15.0	25.0	5.3	1.0	47.2	11.9	2.4
No Treatment (n=10)	Normal	4.9*	6.5*	9.0*	1.4*	25.6*	2.6*	16.3*
No Treatment (n=10)	Malaria	16.9	25.3	5.0	0.8	56.5	12.2	2.3

+Melezitose (1 mg/mouse) administered iv daily for 3 days.

Statistical analysis by the Student "t" test was employed using a 95% confidence level (*indicates statistical significance). Values are expressed as mean values for sake of clarity.

Table 4: Comparative organ uptake of ^{51}Cr-labeled normal or malaria-infected red cells in control and RE depressed mice*

Group	Red Cells Injected	Blood Activity %ID/ml	Liver g	Liver %ID+	Lung g	Lung %ID	Spleen g	Spleen %ID
Tween	Normal	64.2	1.0	4.5	0.1	1.5	0.06	1.0
Methyl Palmitate	Normal	56.9	1.1	4.4	0.1	1.2	0.08	1.5
Tween	Malaria	30.3	1.1	19.8	0.1	1.2	0.08	23.3
Methyl Palmitate	Malaria	34.9	1.2	17.8	0.2	1.5	0.09	17.7

*Values are expressed as means and are derived from 5 to 6 mice per group. Standard errors are not presented for reasons of clarity. Data were obtained six hours following intravenous administration of labeled red cells.

+Percent injected dose per total organ.

presented. Extensive studies were also attempted to label malaria-infected red cells derived from animals with higher degrees of parasitemia. These studies were uniformly unsuccessful due to the increased fragility of the cells.

It is apparent that malaria-infected red cells are indeed rapidly phagocytized in normal animals and that some additional enhancement occurs in RE-stimulated groups. Attempts will be made in the future to increase the rate of phagocytosis and increase lysosomal activity to enhance control of the parasite.

B. Influence of Chemotherapeutic Agents in Combination with RE Altering Agents upon RES Function during Malaria Infection

The recent ability to enhance the phagocytic and immunologic response of the RES by the administration of glucan has opened a new avenue for the investigation of the role of the RES in physiological and pathological conditions.

Similarly, the employment of methyl palmitate, in emulsified form, to induce hypophagocytosis and immune suppression also allows a further evaluation of the role of the RES in physiopathological events.

The importance of the RES in malaria, while long recognized and implied, has never been fully documented or demonstrated. Gobel and Singer (Ann. N.Y. Acad. Sci. 88:149, 1960) have implied that a therapeutic effect of anti-malarial drugs requires the presence of an intact and functional RES for the destruction of the malarial parasite. This finding has also been stressed by

Bliznakov (*Adv. Exp. Biol. Med.* 15:315, 1971). On this basis, we investigated the influence of antimalarial drugs alone and in combination with RE stimulants and depressants on the degree of parasitemia as well as the survival and prolongation of life span.

The principal stimulants employed were glucan; the RE depressant, methyl palmitate. Melezitose, a water-soluble trisaccharide which possesses RE stimulatory activity, was also evaluated for its effects on a malaria infection. It was anticipated that the administration of Quinacrine in combination with RE stimulants would result in increased survival, reduced parasitemia and a prolongation in survival time. In contrast, it was our anticipation that the administration of RE depressants might well nullify the effectiveness of antimalarial agents.

However, it was found that therapeutic treatment using a combination of an RES activating agent with an anti-malarial drug did not enhance host resistance to malaria, i.e., no significant alteration in malarial parasitemia or mortality was observed. Similar results were demonstrated using an RE depressant agent.

In addition, the prophylactic treatment using both glucan and chloroquine neither enhanced survival nor decreased the percent parasitemia. Since it has been suggested that the RE hyperplasia induced by glucan results in a hypofunctional cell, an attempt was made to find a RE stimulant which did not produce a hypofunctional cell. It was found that melezitose, an RE

stimulant, did indeed decrease the malarial parasitemia when administered prophylactically (Table 5).

Diethylstilbestrol (DES) was also employed as an RES stimulant and compared to other RE stimulants. DES, at a dose of 125 μg/mouse, induced RE stimulation as denoted by an 8-fold increase in phagocytosis. This increase in phagocytic activity is also associated in our preliminary studies, with decreased parasitemia and lethality (Tables 6 and 7). The previous inability to observe a protective effect of estrogen with high doses of estrogen may well be related to the concomitant activation of the adrenal cortex and induced lympholytic activity.

C. The Effect of Anti-Lymphocyte Serum on a Malaria Infection

Sheagren and Monaco (Science, 164:1423, 1969) have reported that rabbit anti-serum to mouse lymphocytes, that is ALS, prolongs the survival of mice infected with *Plasmodium berghei*. These investigators pointed out that the immune response of the host to the parasite may contribute to the death of animals with acute malaria infections. The observations that ALS can delay the consequences of malaria infection does not support the existing concept that macrophages are an important host-defense system in malaria infections, as we have previously demonstrated that ALS is cytotoxic to the Kupffer cell and, indeed, RE depression is induced by acute or chronic ALS

Table 5: Effect of melezitose+ on P. *berghei* parasitemia and mortality

Day	Parasitemia (%) Saline	Melezitose
2	5.5	4.7
3	13.4	6.7*
4	16.1	10.0
7	17.8	15.3
8	29.8	13.8*
9	21.5	15.2*
10	38.2	24.2*
11	54.9	32.8*
14	-	28.7
15	-	20.5
16	-	14.4

+Melezitose (1mg/mouse administered iv daily for 5 days. All animals inoculated 24 hours following last injection with 1 x 10^7 *P. berghei* infected erythrocytes.

Mean survival time (MST) was 10.5 days for saline treated animals and 15.8 for melezitose treated animals.

*Statistically significant (P<.05)

Table 6: Effect of diethylstilbestrol (DES) pretreatment on _P_. _berghei_ infection[+]

Day	Parasitemia (%) DES (n=9)	Control (n=7)
1	1	1
2	1	3.3
3	3.7*	21.4
4	9.0	12.3
5	12.7	11.4
6	13.4	18.3
7	19.4	20.9
8	17.4*	42.6
9	27.4	43.1
10	14.8*	26.0
11	-	45.2
12	29.0	42.1
13	20.7*	43.4
14	24.0	-

[+] DES was injected intramuscularly at a dose of 125 ug for 5 days prior to infection. _P_. _berghei_ inoculum contained 1 x 10^7 parasitized erythrocytes given intravenously.

*Significant at the 95% confidence level.

Table 7: Effect of diethylstilbestrol (DES) pretreatment on _P. berghei_ infection.

Days after _P. berghei_ Inoculation	Mortality % DES (n=9)	Control (n=7)
1-9	0	0
10	11.1	57.1
11	33.3	57.1
12	33.3	57.1
13	44.4	71.4
14	55.5	100
15	100	

administration (Pisano et al., Adv. Exp. Med. Biol. 15:237, 1971 and Patterson et al. Proc. Soc. Exp. Biol. Med. 135:831, 1970). Sheagren postulates that by impairing the immune response with ALS, a transient benefit to the host is derived in that immunosuppression permits longer circulation of infected erythrocytes and, thus, a prolongation of survival. Since the degree of parasitemia was less in the ALS-treated animals than in the controls, Sheagren and Monaco also postulate the ALS may have intrinsic antiparasitic effects. This latter concept may be more correct since Brown et al. (Nature, 219:292, 1968) have indicated that *P. berghei* infections are enhanced in thymectomized rats. Brown et al. postulated that cell mediated immunity plays a role in altering the course of malarial infections.

In view of the possible importance of such observations of Sheagren and Monaco that the immune response initiated in malaria-infected animals contributes significantly to death of the animals, studies are currently underway and will continue to determine the contribution of macrophage and lymphoid cells to host response to malaria.

Our initial studies denote that antilymphocytic serum, when given intraperitoneally, does not have a significant effect on the degree of parasitemia, nor does it exert a significant effect on mortality. However, in a preliminary study, when ALS is given intravenously, a profound enhancing effect on the degree of parasitemia is observed (Table 8). These studies will continue and are suggestive that impaired hepatic function,

Table 8: Effect of ALS administration on P. berghei[+] infection

	% Parasitemia	
Day	Saline (n=4)	ALS (n=4)
2	5.9	12.7
3	8.9	36.4*
4	21.0	58.0*
5	34.1	73.2*

[+] P. berghei inoculum contained 10^7 parasitized erythrocytes and was administered iv.

*Significant at the 95% confidence level.

which we observed to occur following intravenous administration of ALS may be a contributing factor in the observed enhancement in parasitemia.

D. Impaired Endotoxin Detoxification as a Factor in Endotoxin (LPS) Hypersensitivity of Malaria-Infected Mice

Since RES activity has been shown to be increased in a malaria infection as evaluated by colloidal carbon (Cantrell and Elko, J. Infect. Dis. 116:429, 1966) or ^{125}I-microaggregated human serum albumin (Sheagren et al. J. Lab. Clin. Med. 75:481, 1970), and since stimulation of the RES is an event usually associated with endotoxin hypersensitivity, the susceptibility of malaria-infected mice to endotoxin was initially evaluated.

The presence of *P. berghei* infection profoundly enhanced the lethality of *S. enteritidis* endotoxin (LPS). The intraperitoneal administration of 100 ug of LPS, which produced no mortality in normal mice, was associated with an 82% mortality in malaria-infected mice (Table 9). Normal mice receiving 500 ug of LPS had a 27% mortality at 24 hours. This in marked contrast to the 100% mortality observed in malaria-infected mice. Sensitivity to endotoxin is also indicated by the finding that a dose of 25 ug of LPS induced in the malaria-infected mice resulted in a 50% mortality, while a 4-fold increase of this dose administered to normal mice did not result in any mortality. The LD_{50} dose in the malaria-infected mice was 25 ug of endotoxin in contrast to an LD_{50} of 1,020 ug in the normal non-infected mice,

Table 9: Effect of P. *berghei* infection* on the lethality+ of S. *enteritidis* endotoxin

Group	Endotoxin ug	Deaths Total	Mortality (%)
Normal	100	0/11	0
"	200	1/10	10.0
"	500	3/11	27.3
"	1000	5/12	41.7
"	1200	10/10	100
Malaria	-	2/20	10.0
"	25	5/10	50.0
"	50	8/10	80.0
"	100	9/11	81.8
"	200	9/10	90.0
"	500	10/10	100.0

*Normal mice received 1 x 10^7 P. berghei infected erythrocytes intraperitoneally, 5 days prior to endotoxin challenge.

+Mortality recorded at 24 hours following endotoxin administration.

indicating an approximate 41-fold enhancement in endotoxin susceptibility in malaria-infected mice.

To determine if alterations in hepatic or splenic endotoxin detoxification ability were responsible for the hypersensitivity to endotoxin, liver and splenic homogenates from malaria-infected and normal animals were assayed for endotoxin detoxification ability by the technique of Trejo and Di Luzio (*Proc. Soc. Exp. Biol. Med.* 136:889, 1971). In essential agreement with the observation of Trejo and Di Luzio who used rat liver and spleen homogenates to inactivate endotoxin, a significant ($P<.001$) endotoxin detoxification ability was present in mouse liver and spleen homogenates (Table 10). The incubation of endotoxin with liver and spleen homogenates prepared from normal mice reduced endotoxin-induced mortality approximately 50% as compared to the mortality observed in the control group which received endotoxin preparation incubated in buffer.

When endotoxin was incubated with liver homogenate prepared from malaria-infected donors, the protective effect of liver homogenates was abolished. Indeed, livers from malaria-infected mice showed a complete absence of endotoxin detoxification ability as mortality patterns were comparable to that observed when endotoxin was incubated with buffer. In contrast, splenic tissue from malaria-infected mice possessed normal detoxification ability as denoted by the reduction in mortality. The enhancement in endotoxin susceptibility in malaria infection

Table 10: Effect of *P. berghei*[*] infection on detoxification of *S. enteritidis* endotoxin by liver and spleen homogenates[+]

Treatment	Tissue Homogenates	Endotoxin (0.1 ug)	Actinomycin D (25 ug)	Deaths Total	Mortality (%)
None	Buffer	+	+	26/29	89.7
None	Liver	+	+	12/29	41.4
None	Liver	-	+	1/27	3.7
None	Spleen	+	+	13/29	44.8
None	Spleen	-	+	1/27	3.7
Malaria	Liver	+	+	11/14	78.6
Malaria	Liver	+	+	1/12	8.3
Malaria	Spleen	+	+	5/14	35.7
Malaria	Spleen	-	+	0/12	0

[*] Normal mice received 1 x 10^7 P. berghei infected erythrocytes intraperitoneally, 5 days prior to the preparation of liver and spleen homogenates.

[+] 4.0 ml of 1% liver and spleen homogenates incubated with 1.0 ug (0.5 ml) of endotoxin in saline at 37°C for 3 hours. After incubation 0.5 ml (250 ug) of Actinomycin D was added, and 0.5 ml of the mixture administered ip to normal mice. Mortality was recorded 48 hours following the administration of the preparation.

may, under certain conditions, be a factor in malaria-induced mortality. Since endotoxin detoxification has been predicated to be a property of hepatic Kupffer cells and not of parenchymal cells, this study adds a new dimension to disturbances in liver function associated with a malaria infection, i.e., an inability to detoxify endotoxin.

Enhanced endotoxin sensitivity in malaria infected mice, as a result of impaired hepatic endotoxin detoxification,has been suggested, under certain conditions, to be a factor in malaria-induced mortality. In an attempt to dissociate the effects of the malarial parasite, a state of endotoxin tolerance was induced.

Endotoxin tolerance was induced in white male mice by 7 daily doses of endotoxin (S. enteritidis) given in increasing increments, i.e., 25 ug, 25 ug, 50 ug, 75 ug, 100 ug and 200 ug of endotoxin. Seventy-two hours after the last endotoxin injection the mice were inoculated (iv) with 1 x 10^7 P. berghei infected erythrocytes. The percent parasitemia was determined on alternate days and mortality was recorded. It was found that endotoxin tolerant mice had a transient protection, lasting approximately 8 days, from the malaria parasite.

It was hypothesized that the endotoxin tolerant condition was broken by blockade of the RES by either the parasite per se or ingestion of altered erythrocytes. To maintain a condition of endotoxin tolerance, alternate day boosts following P. berghei

inoculation, using either 200 ug of endotoxin or 10 ug of endotoxin were employed in subsequent experiments. However, using an endotoxin boosting dose of 200 ug enhanced malaria parasitemia and a 10 ug boosting dose had essentially no effect.

E. Bactericidal Activity of Peritoneal Macrophages from Malaria-Infected Mice

Previous studies have demonstrated that a relationship exists between the functional expression of the reticuloendothelial system and the response of animals to endotoxin. Numerous investigators have observed that macrophages obtained from endotoxin-sensitive animals have an increased bactericidal activity. Therefore, since a malaria infection induces a state of enhanced endotoxin sensitivity and since macrophages have been demonstrated to be intimately involved in a malaria infection, the bactericidal activity of macrophages from malaria infected mice was evaluated. Peritoneal macrophages were obtained from normal and malaria infected mice by injecting 5.0 cc of TC199 into the peritoneal cavity and then withdrawing this fluid after approximately one minute. Viability of the macrophages was determined using either trypan blue of 1% eosin. The macrophages were then incubated with S. aureus for varying periods of time. At specific times aliquots were removed from the incubation tubes and plated on agar. An identical volume of the culture medium containing macrophages and bacteria was lysed in saponin to allow a determination of

the numbers of viable intracellular bacteria (Table 11). It was demonstrated that peritoneal macrophages from malaria infected mice had a significantly enhanced bactericidal activity when compared to peritoneal macrophages obtained from non-infected mice. Therefore, it may be suggested that the enhanced bactericidal activity of macrophages from endotoxin hypersensitive animals, including malaria infected animals, may be due to utilization of intracellular enzymes for bacterial degradation in preference to endotoxin detoxification. Recent evidence (Bullen et al., Immunol. 20:391, 1971) has demonstrated that iron compounds enhance bacterial virulence. Supportive evidence can be seen by the enhanced virulence of bacteria in individuals with hemolytic anemia. Therefore, since malaria induces a state of anemia with an increased deposition of hemozoin, the iron containing malarial pigment in macrophages, studies will be conducted to evaluate the role of this hemozoin in a malaria infection.

5. Conclusions

It may be condluced from this research that the parasitized erythrocyte is indeed recognized as "foreign", as evidenced by its enhanced clearance rate, and that the macrophage is intimately involved in this event as demonstrated by the tissue distribution of the ^{51}Cr-labeled erythrocytes among the major RE organs.

Table 11: Bactericidal activity of peritoneal macrophages from malaria infected mice

Peritoneal Cells	State of Cells	Bacterial Colonies/Plate 0	60	120 minutes
Normal	intact	67	76	62
Malaria		5	6	5
Normal	lysed	76	44	72
Malaria		3	2	3

Peritoneal macrophages (2 x 10^6) incubated at 37°C with 0.5 x 10^6 S. aureus on a Dubnoff shaker at 37°C.

Aliquots (0.1 ml) of macrophage and bacteria suspension removed at 0 - 60 - 120 minutes intervals and plated on agar.

One ml (1 ml) aliquots removed at specified time intervals and subjected to saponin (5%) to induce lysis of macrophages and then plated on agar to determine viable intracellular bacteria.

The enhanced parasitemia seen with intravenous administration of ALS we feel is a reflection of its cytotoxic action on hepatic Kupffer cells in addition to its lymphocyte cytotoxic activity.

Enhanced endotoxin sensitivity and impaired hepatic endotoxin detoxification has been demonstrated in malaria infected mice. This finding demonstrates impaired macrophage function in association with the malaria infection.

In addition to the above studies on the role of the macrophage on the immune response and its influence on malaria, studies on the role of the macrophage on malaria induced immunosuppression as well as the role of the macrophage in the development of malaria induced anemia have been initiated.

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13 ABSTRACT

The purpose of the research described in this report was to determine the role of the reticuloendothelial system (RES) in malaria infectio. The primary hypothesis which was evaluated is that the macrophage is an essential element in host defense to malaria.

Using ^{51}Cr-labeled erythrocytes it was demonstrated that Plasmodium berghei infected erythrocytes were removed from blood at a significantly accelerated rate than normal non-infected erythrocytes. Neither stimulation, nor depression of the RES altered the phagocytic clearance of the labeled erythrocytes. However, certain RE stimulants such as diethylstilbestrol and melezitose, did significantly reduce parasitemia and mortality.

Utilization of the antimalarial agents quinacrine and chloroquine with the RE stimulant, glucan and RE depressant, methyl palmitate, demonstrated no functional synergism between the RES and anti-malaria drugs - an hypothesis which had been advanced by several investigators.

Since RES activity has been shown to be increased in a malaria infection and since stimulation of the RES is an event usually associated with endotoxin hypersensitivity, the susceptibility of malaria-infected mice to endotoxin was evaluated.

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Malaria infected mice manifested a 40-fold increase in endotoxin susceptibility. While liver and spleen homogeantes from normal animals possessed a significant endotoxin-detoxification ability, liver homogeantes prepared from malaria infected mice did not demonstrate endotoxin detoxification ability. The endotoxin detoxifying ability of splenic homogenates prepared from malaria infected donors was however normal.

These findings indicate that endotoxin hypersensitivity in malaria infected mice may be due to an impairment in hepatic endotoxin detoxification. Since endotoxin detoxification by liver has been predicated to be a function of Kupffer cells, the loss of endotoxin detoxifying ability in malaria infected animals adds a new dimension to macrophage functional alterations in malaria.

AD ____________

FACTORS INFLUENCING THE PATTERN OF THE IMMUNE RESPONSE (U)

ANNUAL PROGRESS REPORT

by

Geoffrey Edsall, M.D.

31 August 1971

(For the period 1 June 1970 to 31 May 1971)

Supported by

U.S. ARMY MEDICAL RESEARCH & DEVELOPMENT COMMAND
Office of The Surgeon General, Washington, D. C. 20315
in cooperation with the Commission on Immunization
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Boston 02130

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SUMMARY

The primary serologic response to a single dose of high potency tetanus toxoid, tested in 77 volunteers in a multiracial low income semi-rural Southern U.S. population showed an exceptionally high proportion of "poor responders", and some suggestive evidence for the possibility of genetically linked immune responsiveness.

Purified diphtheria toxin was largely but not completely detoxified by glutaraldehyde aggregation. When aggregation was terminated by addition of lysine, the resulting antigen induced mostly IgM-type, hemagglutinating antibodies; termination by sephadex filtration yielded an antigen inducing primarily an IgG type response. Tetanus Toxoid prepared from column-purified toxin was only minimally enhanced in potency by Al $(OH)_3$ or $AlPO_4$-adsorption.

In a search for possible "natural" tetanus antibodies, a group of 77 isolated Amazonian aborigines were tested for detectable serum tetanus antitoxin levels. One had a minimal titer (0.0025 unit/ml). More completely isolated groups are needed for further studies.

The "principle of uncertainty" applies to potency tests of adsorbed toxoids based on serial dilution, since this would automatically shift the dissociation of the toxoid, perhaps especially in the presence of buffered saline. An experiment to test the acceptability of distilled water disclosed no significant difference from buffered saline in this respect. These studies also indicated an effect of sex and injection route on the immune response.

An earlier study, now included in this report, demonstrated competition between tetanus and diphtheria toxoids.

FOREWORD

This report summarizes work performed under Contract DA-49-193-MD-2144 (1 June 1970-31 October 1970) and Contract DADA17-71-C-1033 (1 November 1970-31 May 1971) with the Office of The Surgeon General, United States Army, and sponsored by the Commission on Immunization, Armed Forces Epidemiological Board. The contract with Harvard University is administered through the Harvard School of Public Health. The work is actually carried out at the State Laboratory Institute of the Massachusetts Department of Public Health in Forest Hills, a few miles from the Harvard Medical-Public Health area. The work has been made possible through the very extensive efforts of William C. Latham, Mrs. Mary Foley, Mrs. Nancy Blake and Ralph Timperi, as well as through the regular and invaluable contributions of more than 30 members of the staff of the State Laboratory Institute, without whose efforts in maintaining the regular services of all types in the Institute, the operation of the contract in question would be impossible.

Valuable and gratifying collaboration with colleagues outside the Institute has included:

Dr. Francis Black, Yale University School of Medicine
Prof. Paul Beaver, Dr. Mark Belsey, and the ICMRT staffs at Tulane University and the Universidad del Valle.

In conducting the research described in this report, the investigators have adhered to the "Guide for Laboratory Animal Facilities and Care" as established by the National Academy of Sciences-National Research Council.

Geoffrey Edsall, M.D. Responsible Investigator	Leo Levine, B.S. Principal Assistant
Superintendent, State Laboratory Institute, Massachusetts Department of Public Health and Professor of Applied Microbiology, Harvard School of Public Health	Chief, Antitoxin Laboratory, Division of Biologic Laboratorie State Laboratory Institute Massachusetts Department of Public Health and Assistant in Microbiology Harvard School of Public Health

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Factors Influencing the Pattern of the Immune Response

Geoffrey Edsall, M.D., Principal Investigator

1. Factors affecting the early primary immune response.

a. Single dose immunization in man

(1) Host factors as determinants of the immune response. The study of the effect of age, sex, race and sibling relationship on the response to a single primary dose of antigen, carried out in a rural parish of Terrebonne, Louisiana (referred to as the "Terrebonne Study") was completed and analyzed, in collaboration with Prof. Mark Belsey of Tulane University Medical School. The study involved the inoculation of a population in a fairly stable rural community, above the age of 14 (since pediatric immunization had been fairly common in the younger age groups) with a high potency tetanus toxoid (Lot AL6D, described in previous reports). Complete records on age, sex, race and family relationship within the community were already available on all subjects, based on another study already underway in the community.

The original aim was to recruit enough families to yield up to 300 subjects. However, for various reasons, some of which are noted below, the total eligible family members recruited was 153, of whom 112 were considered acceptable for inclusion in the study. Of these, 40 more were excluded either because of high Day-7 antitoxin titers, or because of detectable Day 7 titers with excessively high antibody rises by Day 28. A few other subjects were dropped because of insufficient bleedings. The 77 subjects included in the final study, although far fewer than was originally intended, gave some suggestive findings.

The distribution of titers at 28 days, in the 77 subjects analyzed, was thought to have a trimodal character (see Table 1). The 71 acceptable subjects bled on Day 56 showed an essentially similar trend. Even more striking, however, was the astonishingly large number of subjects (at both bleedings) with either negative or minimal titers (Code 0 or 1 in the table). These results represent the poorest overall response yet seen in any of our studies. Because the subjects in this study live in an area subjected to genuine poverty, with what may be very poor average nutritional level, it is tempting to suggest that their relatively poor response was due to malnutrition. Some other measure of immune responsiveness (e.g. antistreptolysin titers, typhoid O-agglutinins, influenza HI antibodies) might provide a useful cross-check on this hypothesis, although interpretation would be uncertain due to insufficient baseline information in this group.

Table 1

Terrebonne Study: Distribution of Tetanus Antitoxin Titers at Days 28 and 56

Titer Code*	Day 28	Day 56
0	26	9
1	13	10
2	2	12
3	4	7
4	3	9
5	1	2
6	5	8
7	5	3
8	5	1
9	3	1
10	3	3
11	4	3
12	1	3
13	1	--
14	1	--
	77	71

*Code: 0 = <.0025 antitoxin unit
1 = .0025 AU
2 = .005AU, etc.

The alternative possibility -- that the toxoid used had fallen in potency from its exceptionally high level of antigenicity in 1966 -- is being rechecked by repeat performance of an International Unit titration for comparison with similar titrations carried out on this toxoid both here and in Holland in previous years.

Table 2 shows the distribution of low, normal, and high titers at 28 and 56 days, by age group; the age groups have been so selected as to give an approximately equal distribution. It will be noted that an exceptionally large number of elderly people were included in the study.

Table 3 shows the distribution of responses by sex. As with age, no significant differences in the distribution of responses can be observed in this limited number of subjects.

Table 4 shows the distribution of responses according to race: Individuals classed as "black" were in general fairly pure blacks, whereas the group designated as "white-Indian" have a well known genealogy in the community, described by the phrase employed. No significant difference based on race could be detected in this very limited selection of subjects.

Figures 1 and 2 show the subject numbers, and antitoxin code titers, in all of the subjects who could be studied in conjunction with one or more relatives. In several instances a close resemblance in antitoxin response was observed (e.g. Subjects 42 and 23, 35 and 36, 109 and 111, and 51 and 54) on Day 28. However, there were also a number of instances in which a very marked discrepancy occurred -- e.g. Nos. 14 and 26, and 46 and 71. Preliminary discussion of these data with a geneticist suggests that they may be quite consistent with the assumption of a genetic determinant, and that further study of this possibility is clearly indicated.

Four CVA's with 3 deaths occurred in the study group, on the 9th, 13th, 63rd and 77th days after the study was begun. Offhand, the incidence of 4 CVA's in a group of 112 subjects appears very alarming, and raises a question concerning the safety of the product employed (despite the fact that only 2 of the 4 episodes occurred within two weeks of the inoculation). However, re-screening of the records of the original 200 subjects under consideration for the study disclosed that 4 of them also died in the 4-month period of observation before the study was begun.

Unfortunately, the first death in the group caused a sharp drop in the enthusiasm of volunteers for the study, which largely explains the inability of the field team to secure as many volunteers as originally planned.

Table 2

Terrebonne Study: Age and Tetanus Antitoxin (TAT) Response

Day 28

Age	TAT Response			Total
	Low	N	High	
15-39	12	14	2	28
40-59	13	6	4	23
60 +	14	8	3	25
Total	39	28	9	76

Day 56

Age	TAT Response			Total
	Low	N	High	
15-39	6	20	1	27
40-59	7	11	4	22
60 +	6	12	3	21
Total	19	43	8	70

Table 3

Terrebonne Study: Sex and Tetanus Antitoxin (TAT) Response

Day 28

Sex	TAT Response			Total
	Low	N	High	
Male	11	4	5	20
Female	28	24	4	56
Total	39	28	9	76

Day 56

Sex	TAT Response			Total
	Low	N	High	
Male	6	9	5	20
Female	13	34	2	49
Total	19	43	7	69

Table 4

Terrebonne Study: Race and Tetanus Antitoxin (TAT) Response

Day 28

Race	TAT Response Low	N	High	Total
White	4	5	3	12
Black	25	16	5	46
White-Indian	9	7	1	17
Total	38	28	9	75

Day 56

Race	TAT Response Low	N	High	Total
White	2	7	3	12
Black	10	27	4	41
White-Indian	7	8	1	16
Total	19	42	8	69

Fig. 1 Terrebonne Study: Relationships vs. Titer
(Code titer shown inside circles or squares)

Day 28

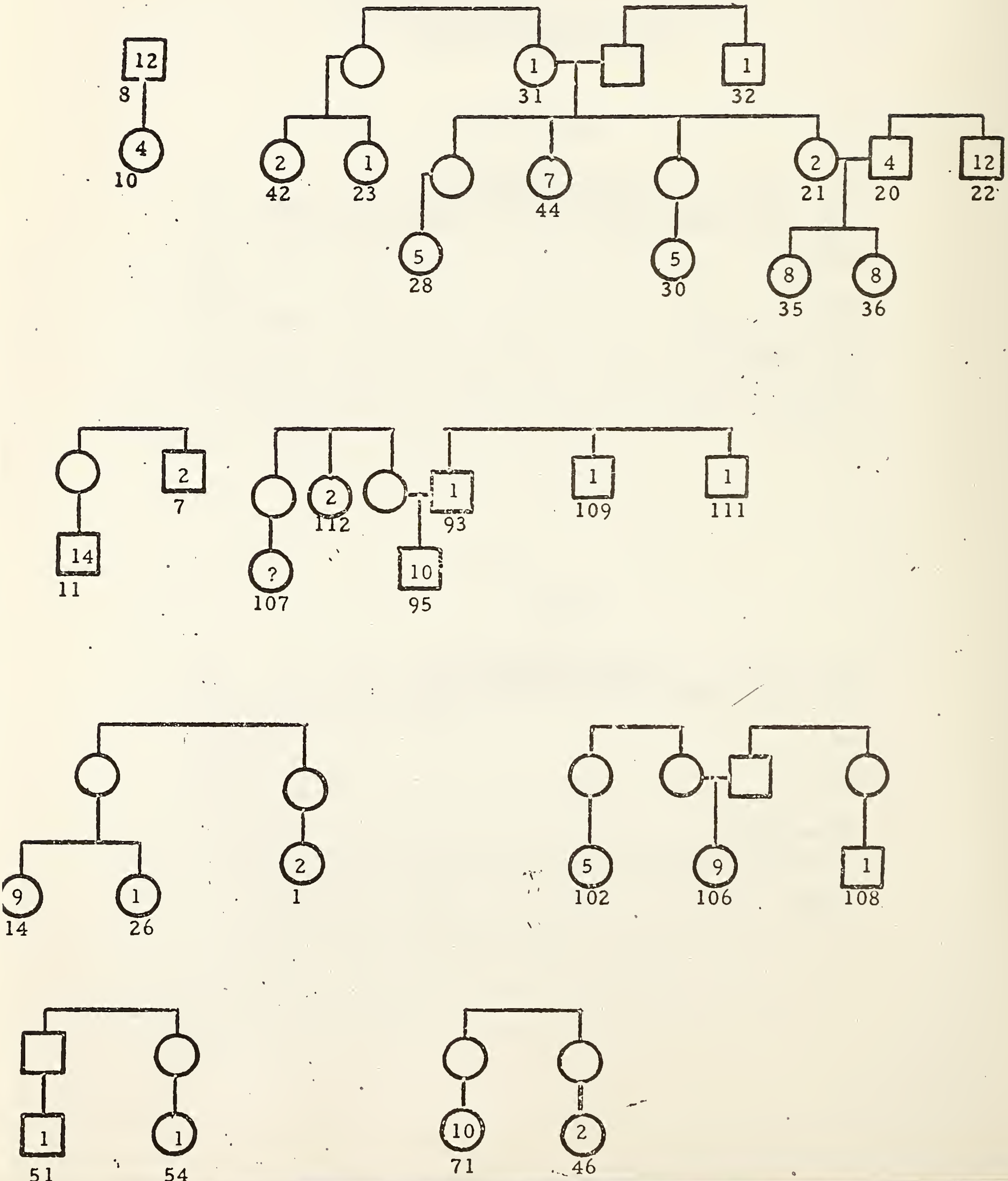
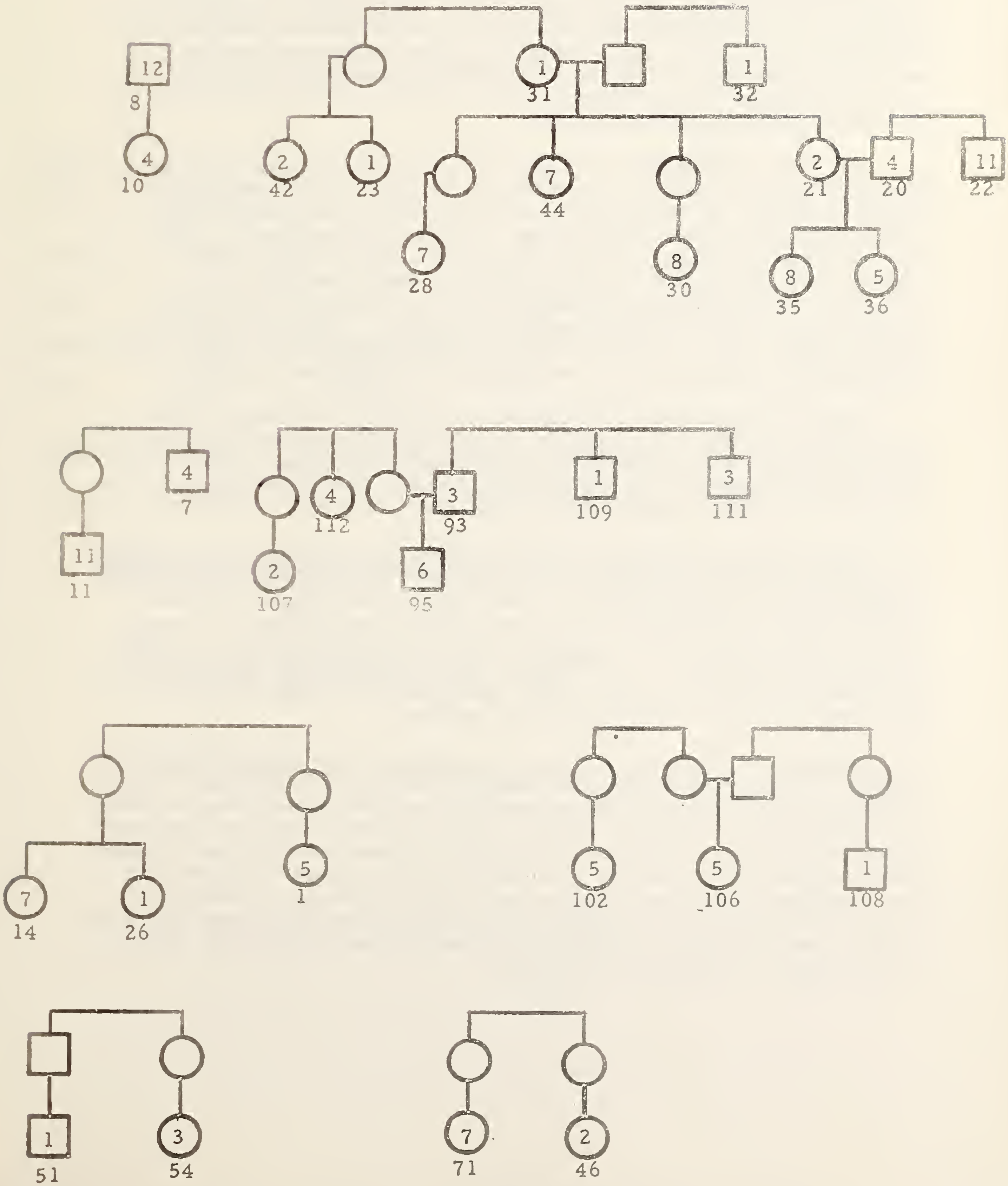


Fig. 2 Terrebonne Study: Relationships vs. Titer

(Code titer shown inside circles or squares)

Day 56



Other studies on the effect of single-dose immunization under defined circumstances are in the planning stages, viz., immunization of sugar-cane workers in the Palmira area, Departamento del Valle, Colombia. In collaboration with the Area Health Service and the Universidad del Valle, 100 or more adult male sugar-cane workers, who run a high risk of tetanus, will be inoculated with a single dose of the high potency tetanus toxoid and titrated one year later for their residual antibody level.

(2) Considerable interest has been aroused by the studies reported by Rey of Dakar, working in collaboration with Triau of the Institut Merieux in Lyon, on the use of a highly concentrated fluid toxoid for single-dose immunization. Through the courtesy of Prof. Rey, we obtained samples of both plain and adsorbed preparations of this type of concentrated toxoid. Table 5 summarizes the data obtained. The Massachusetts preparations used for comparison were a bulk concentrated fluid preparation, diluted to 50Lf for the flocculation test, and a standard aluminum phosphate-adsorbed preparation in routine distribution, for the comparison of adsorbed toxoids.

From the viewpoint of a single-dose toxoid, the potency of the Merieux preparation (adsorbed) at 191 IU/ml may be compared with our Lot AL15 ("high-high") which in three assays gave values of 563, 499, and 510 IU/ml. It contained only 50 Lf/ml of toxoid antigen, against 720 Lf/ml in the Merieux toxoid.

2. Role of first vs. second dose in determining antibody level attained following 2 doses of antigen. In August 1970 the Principal Investigator visited Cali to lay on a protocol and inspect the proposed site for carrying out this study. The site chosen, the male prison at Popayan, had the full approval and support of the Prison Director and the other authorities concerned. Due, however, to a change in the scientific personnel involved, it has not been possible to implement the study, but plans for doing so in 1971-72 are underway.

3. Efficacy of 0.1 ml vs. 0.5 ml of a third dose of Tetanus-Diphtheria Toxoid. At the fourth Army Post where an effort has been made to carry out this study, the Chief of the Hospital has been endeavoring to secure volunteers, but with little success. It is probable that the study may be more effectively carried out through collaboration with Capt. Michael Benenson, M.D., USA, who has a collection of sera taken from arrivals and departees from South Vietnam, which should provide the essential answer to the question -- namely, Is a soldier going to a high-risk area such as South Vietnam adequately protected against diphtheria? This study should be completed in 1971-72.

Table 5

Comparative Tests on Tetanus Toxoids from Institut Merieux and Massachusetts State Laboratory Institute

Toxoid Source	Lot No.	Type	Lf/ml	Kf @ 50Lf min*	IU/ml	IU/Lf
Merieux	36	Fluid	720	90	(1)	(1)
Mass.	LP265	Fluid	(50)	20		
Merieux	36	Adsorbed(2)	720	-	191	0.26
Mass.	AL14	Adsorbed	10	-	82	8.2

(1) No significant dosage-response regression obtained.

(2) Only about 10% was adsorbed. 646 Lf/ml found in supernatant

* Kf = flocculation time, a useful inverse indicator of the integrity of the antigen after detoxification, etc.

4. Immunity to Diphtheria. Following the advice of Dr. J. D. van Ramshorst of Utrecht, who has extensively studied and modified a highly sensitive Czech technique for cell-culture titration of diphtheria toxin and antitoxin, this method is now being tried out at the Institute as a possible replacement for the rabbit test, which suffers at present from an extraordinary shortage of rabbits with skins suitable for use in the test.

5. Effect of Aggregation on the Antigenicity of Purified Diphtheria Toxoid. Last year's report mentioned continuing difficulties in the preparation of a homogeneous diphtheria fraction sufficiently pure to be considered satisfactory for this study. However, by modifying the conditions described by Pappenheimer for a second-step purification of diphtheria toxin by elution from a DEAE-cellulose column, satisfactory preparations in adequate working quantities have now been obtained. Since glutaraldehyde (GA) did not appear to aggregate diphtheria toxoid, studies have been focused on attempts to achieve both detoxification and aggregation of toxin with suitable concentrations of GA. At a mol ratio of about 1,000 GA/1 toxin, the reaction being stopped after one hour by 0.5 mol of lysine, toxicity was reduced over 99.9%, but not totally eliminated. Yet this ratio is high when compared to that which has been used successfully by others in enhancing antigenicity of protein antigens. All variations in mol ratio tried to date, with purified toxin, have appeared to contain at least small traces of residual toxicity. Further studies are still needed to determine the optimal concentration of GA, the optimal amount of lysine for terminating the reaction, the optimal reaction time, etc. Additionally, the possibility of primary detoxification with GA, followed by a period of treatment with a low concentration of formaldehyde, appears to warrant consideration.

Our earlier trial of the effect of GA aggregation on immunogenicity was repeated except that the action of GA was arrested by the addition of lysine instead of filtration through Sephadex G50. The product (GL) was tested in guinea pigs using two response parameters, serum titration by HA and survival time following challenge. A regular adsorbed diphtheria toxoid was used as a control (C). The GA-Sephadex (GS) material was also tested.

In serum titration the decreasing rank order of materials under test was GL, C, GS.

In survival time of challenged guinea pigs the order was C, GS, and GL. This finding was investigated by fractionation of individual animal sera with Sephadex G200. The HA titers were done on each 'γG' and 'γM' fraction. With C and GS, the agglutinating activity was primarily 'γG'. With GL it was mainly 'γM'. Thus the lack of correlation between the agglutinating activity and the neutralizing activity appears to be related to the class of globulin in which it is found.

6. Purification and characterization of Toxins and Toxoids.

Column purified tetanus toxin.

Further immunogenicity testing in mice was carried out on the formaldehyde-detoxified fraction of tetanus toxin described in previous reports. It was confirmed that adsorption on aluminum phosphate or aluminum hydroxide did not result in the increased potency that one generally expects from these adjuvants. The data are illustrated in Fig . 3 and given in the caption. Three aliquots of a column purified preparation, Lot 134, were tested at a concentration of 10 Lf per ml; a standard adsorbed toxoid, Lot AL 14, was included as a reference. The plain toxoid without adjuvant, was designated 134A. Lot AL 134 contained 4 mg/ml $AlPO_4$ and Lot AH134 contained 4 mg/ml $Al(OH)_3$. The assay showed potencies of 28, 35, and 40 IU/ml, respectively, values that do not differ significantly.

Contrast with fractionation of toxoid.

Earlier we had used the same chromatographic methods on tetanus toxoid that had been detoxified in the crude state. The most actively flocculating fraction obtained was tested in the fluid state at 100 Lf/ml parallel with the same concentration of our routine ammonium-sulfate-processed toxoid, Lot LP243. At the same time we tested two preparations designed for single-injection immunization, Lots AL23 and AL26, which had 100 Lf/ml and 7.72 mg $AlPO_4$/ml. The dose-response lines are shown in Fig. 4 and the results in IU/ml are given in the figure caption. It is of interest that the plain toxoid fraction (Lot CPT130B) at 100 Lf/ml had 29 IU/ml whereas the plain toxoid of Fig. 3 that was isolated as toxin had 28 IU/ml at only 10 Lf/ml. These data confirm our earlier impression that column purification of tetanus toxin, followed by detoxification, yields a preparation of considerably higher immunizing potency per Lf unit than has generally been obtained when detoxification preceded, rather than followed purification.

The failure of aluminum salts to enhance the antigenicity of this preparation is of interest, and recalls the similar finding recently observed with cholera toxoid. Relevant information, such as the isoelectric point of the detoxified column-purified toxin, will be sought.

Effect of formaldehyde and antigen concentration during detoxification of purified tetanus toxin on the immunogenicity of the product.

Ten preparations of toxoid were made from aliquots of the same fraction of purified tetanus toxin involving 4 concentrations of formaldehyde and two concentrations of toxin as tabulated on the graph of Fig. 5. The reaction mixtures were retained for one week at 37°C, 2 weeks at 5°C and then dialized to remove free formaldehyde.

Caption for Fig. 3

Immunogenicity of 3 adjuvant forms of a single fraction of tetanus toxin detoxified with 10mM of formaldehyde and diluted to a concentration of 10 Lf/ml.

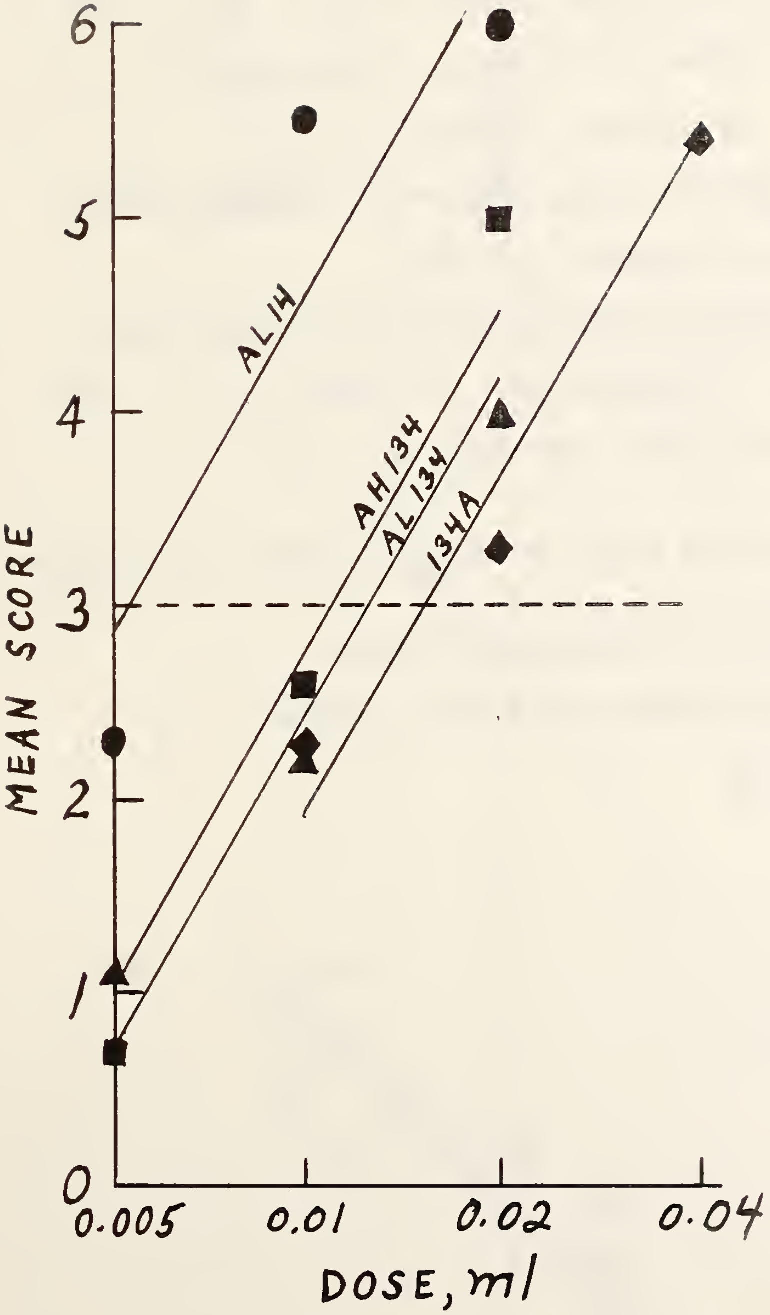
1. 134A: Plain toxoid, no adjuvant. 28 IU/ml
2. AL134: 4 mg/ml $AlPO_4$. 35 IU/ml
3. AH134: 4 mg/ml $Al(OH)_3$. 40 IU/ml

Lot AL14 is the standard 4 mg/ml $AlPO_4$, 10 Lf/ml, 82 IU/ml.

Each point is the mean response of 10 mice.

The dashed line indicates the median response.

FIGURE 3


6
5
4
3
2
1
0
MEAN SCORE
AL14
AH134
AL134
134A
0.005
0.01
0.02
0.04
DOSE, ml

Caption for Fig. 4

Immunogenicity of 4 tetanus toxoids in mice:

1. CPT130B: Column purified in the toxoid state.

   100 Lf/ml, no adjuvant. 29 IU/ml.

2. LP243: Ammonium sulfate purified in the toxoid state.

   100 Lf/ml, no adjuvant. 22 IU/ml.

3. AL23: Ammonium sulfate purified in the toxoid state.

   100 Lf/ml, 7.72 mg $AlPO_4$/ml. 599 IU/ml.

4. AL26: Same as AL23. 547 IU/ml.

Lot AL14 is included as the standard (10 Lf/ml, 4 mg $AlPO_4$/ml, 82 IU/ml).

Each point is the mean response of 10 mice.

The dashed line indicates the median response.

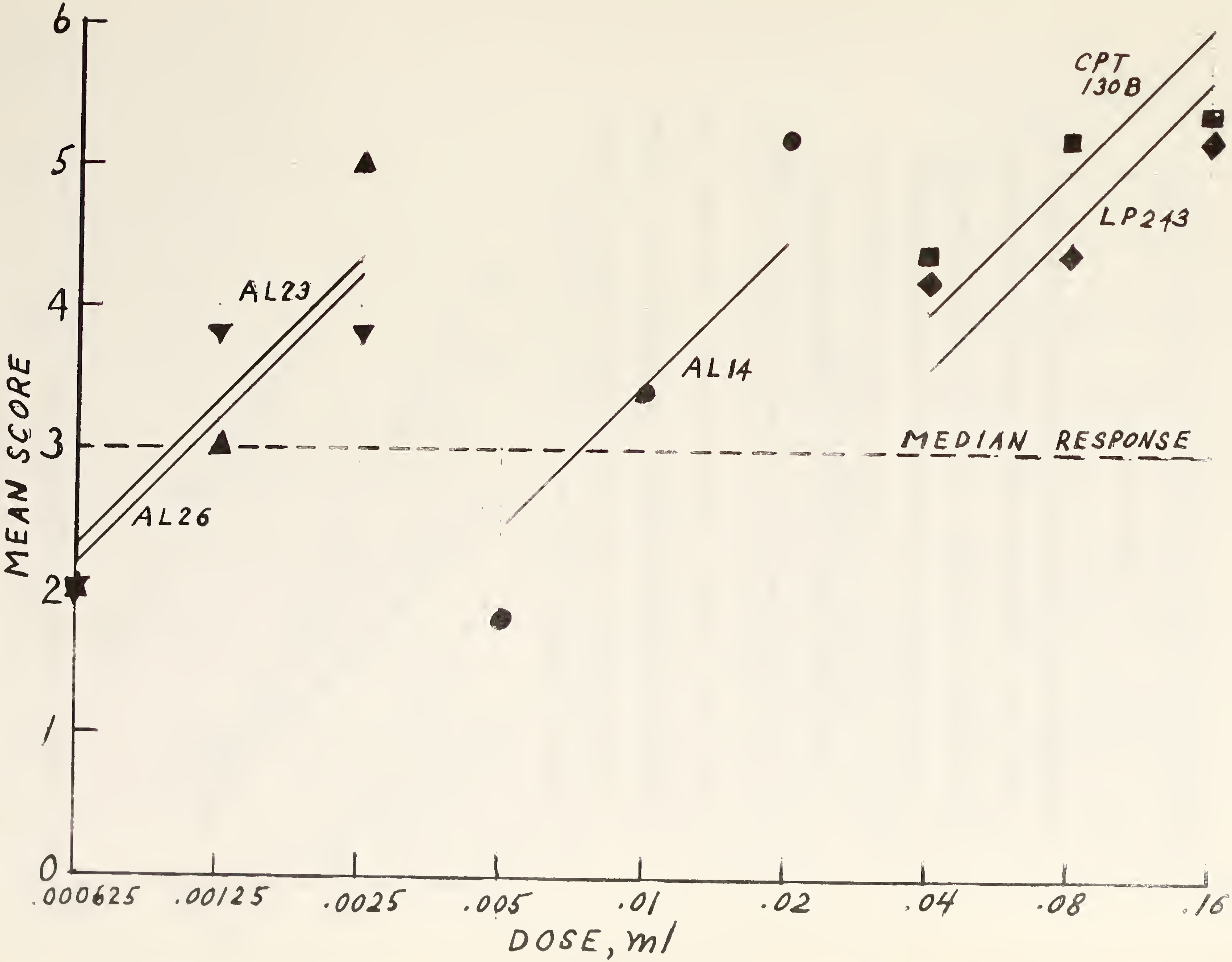
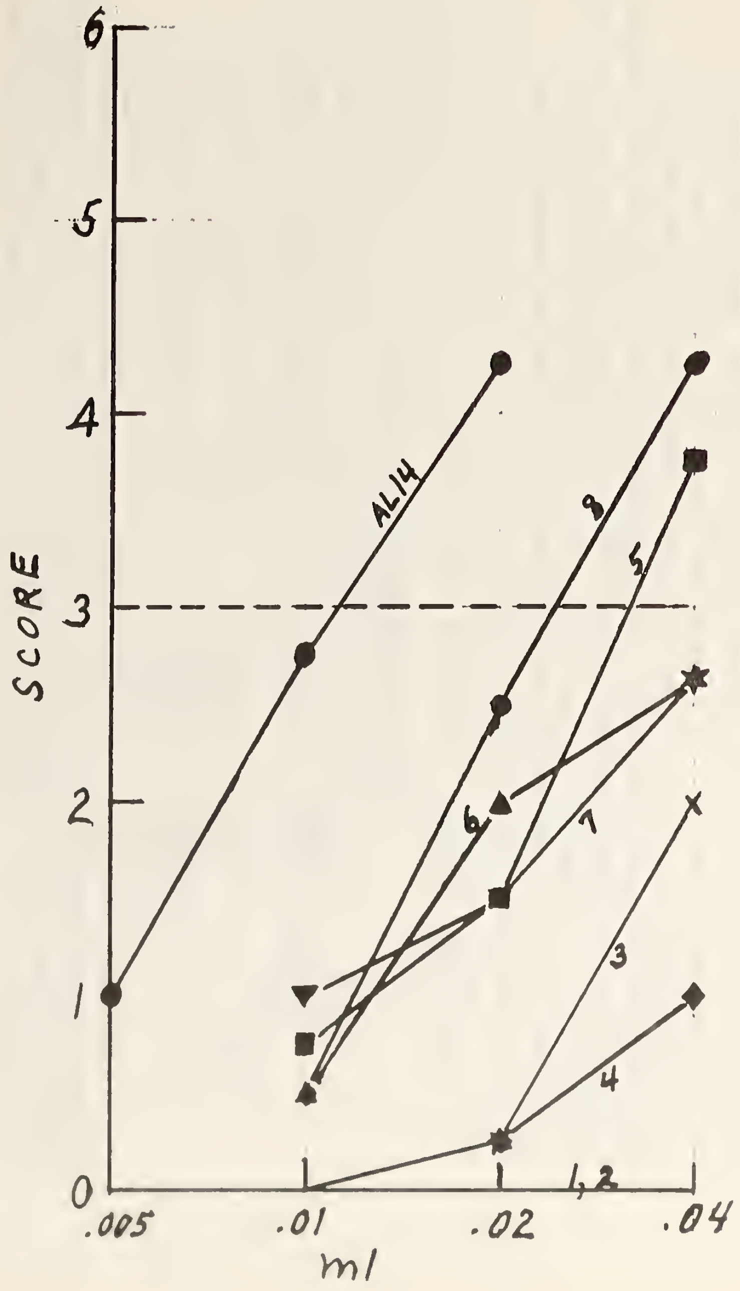

MEAN SCORE
0
1
2
3
4
5
6
DOSE, ml
.000625
.00125
.0025
.005
.01
.02
.04
.08
.16
AL23
AL26
AL14
CPT 130B
LP243
MEDIAN RESPONSE

FIGURE 4

Caption for Fig. 5

Immunogenicity in mice of a single lot of column purified tetanus toxin detoxified with 8 different concentrations of formaldehyde. The number (1 to 8) on each dose-response curve corresponds to the box number on the accompanying diagram which gives the concentration of formaldehyde and of toxin protein and the potency in International Units per ml by comparison with the standard lot AL14 (82 IU/ml).

Each point is the mean response of 8 mice. The dashed line indicates the median response. (No responses were obtained for lines 1 and 2. Lines 3 and 4 could not give a potency estimate because of non-linearity and non-parallelism).



TOXIN CONCENTRATION
Lf/ml

FORMALDEHYDE CONCENTRATION mM/ml	50	100
40	#2 <20 IU/ml	#1 <20 IU/ml
20	#4 <20 IU/ml	#3 <20 IU/ml
10	#6 25 IU/ml	#5 29 IU/ml
5	#8 36 IU/ml	#7 25 IU/ml
2.5	#10 TOXIC	#9 TOXIC

FIGURE 5

Each preparation was then diluted to 10 Lf/ml and tested for potency in mice against our standard Lot AL14. Lots 9 and 10 were toxic for mice. The dose-response curves of the 8 non-toxic preparations have been drawn in Fig. 5, numbered to correspond to the numbered boxes of the accompanying table. It is seen that the highest concentration of formaldehyde, 40 mM/ml, resulted in no detectable response at either toxoid concentration (50 or 100 Lf/ml) during treatment. Curves 3 and 4, representing 20 mM/ml of formaldehyde, did not provide an estimate of relative potency due to non-linearity and non-parallelism. The remaining 4 curves were linear and parallel at the 95% confidence level and again confirmed that the milder the treatment, the greater the potency.

Reversion of toxicity.

We have tested our toxoid over a period of 6 months in guinea pigs using doses of 500 Lf, without indications of toxicity. A colleague (Dr. Garcia) has tested our first preparation in rabbits given 100 Lf approximately one year after production, with no evidence of toxicity. Therefore our limited experience to date would indicate that reversion does not occur, unlike the findings reported by the Wellcome Laboratories in the case of diphtheria toxoid that was purified as toxin.

7. Bioassay of Adsorbed Tetanus Toxoids in Mice.

These assays require low doses of adsorbed toxoids in order to approximate the median effective dose in mice. Injection of such low doses (0.001 to 0.01 ml) involves high dilution of preparations that are at the proper concentration for clinical use. There has often been concern that the process of dilution may result in qualitative changes either by elution of antigen or by solubilization of the aluminum salt. It was considered on theoretical grounds that phosphate buffered saline (PBS) would be more likely to elute a protein antigen from a mineral adsorbate than distilled water (DW). An experiment was designed to test this and at the same time to compare the efficacy of the SC versus the IP route and the immunizability of male versus female Charles River CD-1 mice.

The design of this experiment was as below:

TETANUS ANTIGENICITY (MOUSE)
SEX, ROUTE, AND DILUENT

MALE

ROUTE DILUENT	SC	IP
PBS	4 doses x 10 mice	3 doses x 10 mice
DW	4 doses x 10 mice	3 doses x 10 mice

FEMALE

ROUTE DILUENT	SC	IP
PBS	4 doses x 10 mice	3 doses x 10 mice
DW	4 doses x 10 mice	3 doses x 10 mice

Charles River Breeding Labs, CD-1 strain, 18-20 gms 14 days of immunization
15 MLD Tetanus Toxin Challenge. Antigen: AL14 10 Lf/ml 4 mg $AlPO_4$/ml
Dose Schedule (ml): 0.0025, 0.005, 0.01, and 0.02 if there was a 4th dose.

Appropriate dilutions of Lot AL14 were made immediately before injection so as to obtain the constant dose volume of 0.5 ml.

Survival time and symptoms of the toxin-challenged mice were scored from 0 to 6 as described by Ipsen and these scores constituted the response metameter in a multiple regression analysis. The relative "potencies" of each treatment were computed as tabulated below, with the arbitrary potency of unity assigned to the male-SC-saline group:

Effect of sex, route, and diluent on the relative potency of adsorbed tetanus toxoid in Charles River CD-1 mice:

RELATIVE POTENCY: MALE, SC, SALINE=1

MALE		
ROUTE		
DILUENT	SC	IP
PBS	1	3.07
DW	1.04	2.25

FEMALE		
ROUTE		
DILUENT	SC	IP
PBS	2.31	3.29
DW	2.39	2.96

Between sexes: t = 1.53; $0.1<p<0.2$

Between routes: t = 1.87; $0.05<p<0.1$

Between diluents: t = 0.26; $p=0.8$

The Student's t tests shown above were formed from the differences between the summed relative potencies for sexes, routes, or diluents (as the case might be) divided by the standard error of such difference.

The P-values, therefore, show the probability that the differences were due to chance. This probability was 80% in the case of the diluents so that either diluent is acceptable. The probability of chance effects was much lower for sex and route, but neither one reached the 5% level. It was therefore decided to repeat the experiment (omitting the diluent variable) at the more ideal dose levels ascertainable from these data. The doses used gave an overall immunity of only 31% which resulted in a higher error than would have obtained at levels nearer 50%. The second experiment, described below, showed 56% overall immunity

SEX AND ROUTE

ROUTE SEX	SC	IP
MALE	3 doses x 10 mice	3 doses x 10 mice
FEMALE	3 doses x 10 mice	3 doses x 10 mice

Mice: Charles River Labs, CD-1 Strain, 18-20 grams, 14 days immunization.
15 MLD Tetanus Toxin Challenge. Buffered Saline Diluent
Antigen: AL14 10 Lf/ml, 4 mg. $AlPO_4$/ml

Dose Schedule (ml): 0.02, 0.04, 0.08 for Male, SC
0.005, 0.01, 0.02 for all others.

The results are given below:

Effect of sex and route on the relative potency of adsorbed tetanus toxoid in Charles River CD-1 mice

RELATIVE POTENCY: MALE, SC=1

MALE			FEMALE		
ROUTE	SC	IP	ROUTE	SC	IP
	1	4.71		3.66	4.71

Between sexes: t=2.36; p=0.02
Between routes: t=3.268; $0.001<p<0.01$

The P-value of less than 0.01 for routes indicates that the IP route in mice gives a greater response to this preparation than the SC route. The P-value of 0.02 for sexes indicates that the females of this source are more immunizable than the males. However, the data indicate that both sexes respond equally when the IP route is used and demonstrate a difference only with the SC route.

6. Competition between two protein antigens in the primary response. Recent studies on cell cooperation have highlighted the fact that in two antigen systems, one antigen may either compete with the other, or be neutral in the system. Therefore, closer evaluation of the possibilities of antigenic competition is again receiving more attention. Mr. Levine has re-examined a study done several years ago on this point and summarized it as follows:

Competition between two protein antigens in the primary response of guinea pigs:

Four groups of 6 guinea pigs were given combined diphtheria and tetanus toxoids, adsorbed (DT) SC. For each group the diphtheria toxoid dose was kept constant at 2 Lf, and the tetanus toxoid dose was varied as follows: 0, 2, 4, and 8 Lf. One Lf of each toxoid is approximately 3µg protein. The adsorbent was 2 mg $AlPO_4$ per dose.

Immune response to the tetanus toxoid was not measured. Three different immune responses to the diphtheria toxoid component were measured in each animal, namely:

(a) Serum diphtheria antitoxin titers by the toxin neutralization (TN) method as tested intradermally in rabbits, referred to the NIH standard diphtheria antitoxin, and expressed as International Antitoxin Units (AU) per ml. Titrations were done at 2-fold intervals starting with 0.015625-0.03125 AU/ml. The geometric mean mid-intervals were coded by the successive integers 0, 1, 2, 3, etc., designated "y", which may be defined as:

$$y = \frac{\log AU + 1.656}{0.301}$$

(b) Serum indirect hemagglutination titers (HA method), referred to the NIH standard diphtheria antitoxin, and expressed as hemagglutinin units (HU) per ml. These titrations were also done at 2-fold intervals starting with 0.0625-0.125 HU/ml. The geometric mean mid-intervals were coded by the successive integers 0, 1, 2, 3, etc., designated "x", which may be defined as:

$$x = \frac{\log HU + 1.086}{0.301}$$

(c) Survival time of the animals following challenge with 100 L+ doses of diphtheria toxin 6 weeks after immunization and 2 weeks after the cardiac blood sampling that provided the serum. The survival times in hours were transformed to a score system (based on a linear regression on log dose of toxin) as follows:

Hrs. survival	<14	14-20	21-29	30-34	45-68	69-119	≥120
Score (z)	0	2	5	7	8	9	10

The results are shown in Fig. 6. The presence of an equal amount of tetanus toxoid (6 µg) resulted in a decrease in all 3 response parameters of the diphtheria toxoid component from the levels found in the absence of tetanus toxoid. When the added tetanus toxoid was double or quadruple that of the diphtheria toxoid, the serum responses to the latter antigen did not decline further but remained essentially

Caption for Figure 6

Fig 6. Three measurements of the primary response of guinea pigs to diphtheria toxoid in competition with tetanus toxoid.

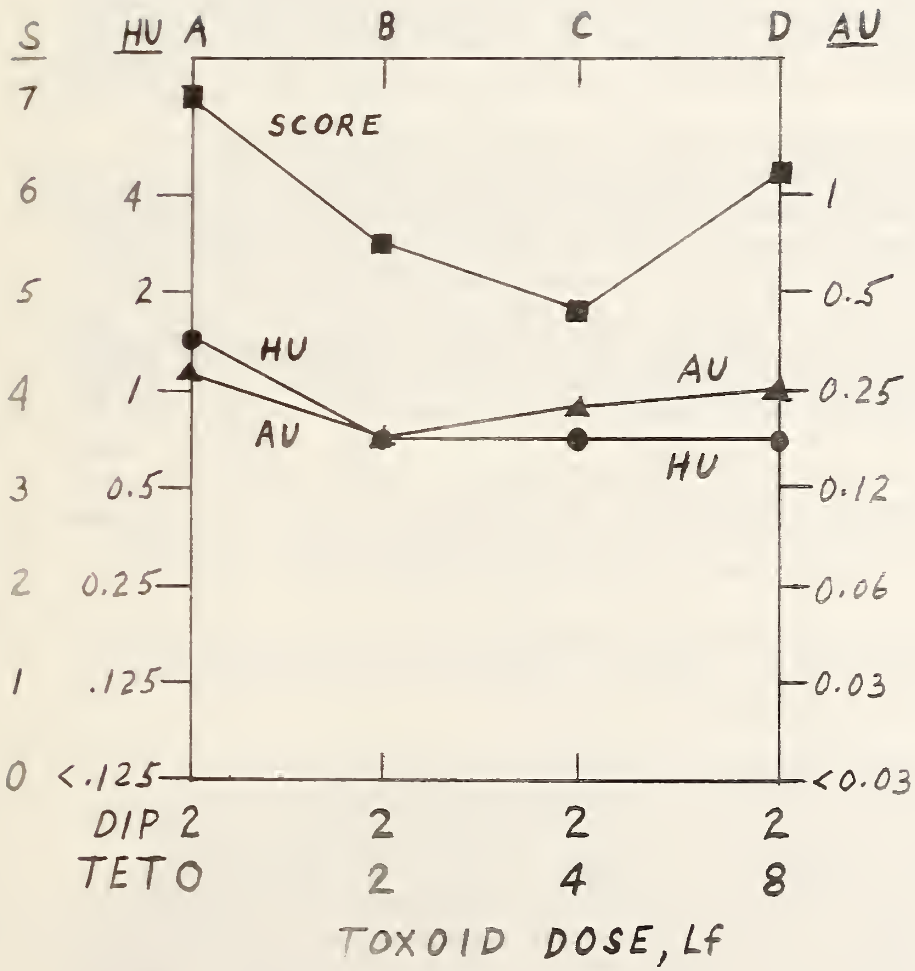
S = Survival time scores of challenged guinea pigs following immunization. See text for details.

HU = Hemagglutinin Units per ml.

AU = Antitoxin Units per ml.

A,B,C,D Guinea pig dosage groups comprising 6 animals each.

FIGURE 6


S
HU
A
B
C
D
AU
7
6
5
4
3
2
1
0
4
2
1
0.5
0.25
.125
<.125
1
0.5
0.25
0.12
0.06
0.03
<0.03
SCORE
HU
AU
AU
HU
DIP 2
2
2
2
TETO
2
4
8
TOXOID DOSE, Lf

constant. The response based on survival time after challenge, however, did decline somewhat when the antigen ratio was 2 and recovered about one-half the total loss when the ratio was 4.

Combined toxoids used clinically for human immunization contain tetanus toxoid in amounts equal to or less than those of the diphtheria toxoid component so that the initial decline of the diphtheria toxoid response from the monovalent state is of practical interest and was further analyzed. This was done by considering the differences of means of the 3 responses between the group A guinea pigs (no tetanus toxoid) and the group B guinea pigs (equal tetanus toxoid). In the case of the indirect hemagglutinating activity, the decline in mean HU/ml from group A to group B was 50%. In the case of neutralizing activity, the decline in mean AU/ml was 37%. In the case of immunogenicity as measured by survival time score the mean decline was 21.4%.

A Student's t-test for the significance of the difference of these means was calculated for each type of response, giving the values 1.50 (9 degrees of freedom) 1.09 (10 df) and 1.54 (10 df) for the HA, TN, and scored responses, respectively. These values are far from 2.23, which they would have to reach to be considered significant at the conventional 5% level. However, when the 3 differences of means were combined, using the reciprocal of the variance of each difference as the weighting factor, a t value of 2.294 with 29 degrees of freedom was obtained, giving $0.02 < P < 0.05$.

The three responses were highly correlated within the 24 guinea pigs. The correlation coefficients and their standard errors between x and z (HU and scores) were 0.79 ± 0.13, between y and z (AU and scores) 0.70 ± 0.16 and between x and y (HU and AU) 0.86 ± 0.11.

The peculiar rise of survival time score with the highest dose of tetanus toxoid may be an artefact, since it is not supported by a similar rise in serum titers.

9. Other Studies. The suggestion has repeatedly been made, that since some individuals with no known history of tetanus have tetanus antitoxin in their serum, that there must be such a thing as "natural" immunity to tetanus. This is an old idea that has lived and died in the past, but keeps being reborn. It becomes important each time a new study group is evaluated for acceptability in a "primary immunization" study, and is found to include a significant number of subjects already carrying tetanus antitoxin -- as, for instance, in the "Terrebonne Study" group above. Therefore an attempt is being made to secure serum samples from aborigines living far from civilization and unlikely to have been exposed to tetanus toxoid immunization. Through the great courtesy and cooperation of Prof. Francis L. Black of Yale University School of Medicine, 77 such

sera were furnished from the Xikrin and Tiriyo tribes of the Amazon jungles, some of which have been described by Black et al in published papers. One such serum disclosed a minimal (0.0025 AU) titer of antitoxin; the other 76 had no measurable antitoxin. The borderline subject came from the tribe which has had occasional contact with the Brazilian Air Force and might therefore have been flown out once for treatment, inoculated at a military hospital or the equivalent. Additional sera are now being sought from other groups working with aborigines in other areas.

10. Presentation of studies supported under this contract: The Principal Investigator presented the essence of the single-dose immunization studies at the X International Congress for Microbiology, held in Mexico City in August 1970 (Abstract attached) and at the III International Conference on Tetanus, held in São Paulo the following week.

The Principal Investigator was also a member of an informal consultation group brought together by the World Health Organization in June-July 1971 to review the existing information on the effect of malnutrition on the immune response, and to plan field studies to further define the situation.

Manuscript in Press: Newell, K.W., LeBlanc, D.R., Edsall, G., Levine, L., Christensen, H., Montouri, M.H. and Ramirez, N. The Serological Assessment of a Tetanus Toxoid Field Trial, Bull. Wld.Hlth. Org.

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13. ABSTRACT

The primary serologic response to a single dose of high potency tetanus toxoid, tested in 77 volunteers in a multiracial low income semi-rural Southern U.S. population showed an exceptionally high proportion of "poor responders", and some suggestive evidence for the possibility of genetically linked immune responsiveness.

Purified diphtheria toxin was largely but not completely detoxified by glutaraldehyde aggregation. When aggregation was terminated by addition of lysine, the resulting antigen induced mostly IgM-type, hemagglutinating antibodies; termination by sephadex filtration yielded an antigen inducing primarily an IgG type response. Tetanus Toxoid prepared from column-purified toxin was only minimally enhanced in potency by $Al(OH)_3$ or $AlPO_4$-adsorption.

In a search for possible "natural" tetanus antibodies, a group of 77 isolated Amazonian aborigines were tested for detectable serum tetanus antitoxin levels One had a minimal titer (0.0025 unit/ml). More completely isolated groups are needed for further studies.

The "principle of uncertainty" applies to potency tests of adsorbed toxoids based on serial dilution, since this would automatically shift the dissociation of the toxoid, perhaps especially in the presence of buffered saline. An experiment to test the acceptability of distilled water disclosed no significant difference from buffered saline in this respect. These studies also indicated an effect of sex and injection route on the immune response.

An earlier study, now included in this report, demonstrated competition between tetanus and diphtheria toxoids.

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	ROLE	WT	ROLE	WT	ROLE	WT

AD____________________

ANTIBODY STRUCTURE AND FUNCTION

ANNUAL PROGRESS REPORT

by

Herman N. Eisen, M. D.

September, 1971

(For the period 1 September, 1970 to 30 August, 1971)

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ABSTRACT

Two mouse myeloma proteins (nos. 315 and 460) that bind Dnp antigens specifically and with high affinity were analyzed with respect to amino acid sequences in variable (V) regions of their light (L) and heavy (H) chains, and the amino acid residues that make-up their specific combining sites. The L chain of protein 315 (L^{315}) has been completely sequenced (residues 1 to 214). Residues 1 to 88 of H^{315} and 1 to 63 of L^{460} have also been determined. Affinity-labeling with various Dnp-bromoacetyl reagents labeled Tyrosine-34 of L^{315}, Lysine-54 of H^{315}, and Lysine-50 of L^{460}. The labeled residue in each case was situated in a hypervariable segment (of the V_L or V_H region); these small segments seem therefore to form the specific combining sites.

BALB/c mice immunized with purified protein 315 made antibodies that reacted specifically with unique determinants (idiotypes) in the protein's Dnp-combining site. The immunized mice also rejected grafts of MOPC-315, the myeloma tumor that produces protein 315; this protein can thus behave as a "tumor-specific transplantation antigen."

To study the strange cross-reaction between Dnp and Vitamin K_3 (2-methyl-1,4-naphthoquinone), antibodies to K_3 were raised by immunizing rabbits with K_3-protein conjugates in which K_3 was covalently attached to protein SH groups via thioether linkage. The same type of reaction was used to prepare K_3-specific solid immunoadsorbants and various K_3-haptens.

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SUMMARY

Primary Structure of Variable Domains of Anti-Dnp Myeloma Proteins.

Two mouse myeloma proteins (nos. 315 and 460) that bind Dnp antigens and haptens specifically and with high affinity offer an almost unique opportunity to establish the elements in primary structure that determine the fine structure and specificity of the ligand-binding sites of these immunoglobulins, of which there is one per Fab domain. The complete sequence of the light chain of protein 315 has been deduced. Residues 1-88 of the heavy chain of this protein, and residues 1-63 of the light chain of protein 460 have also been determined. Affinity-labeling of the intact proteins with Dnp-bromoacetyl derivatives of lysine, ethylenediamine, and other small molecules has led to exclusive labeling of residues that are now established as tyrosine-34 in L^{315}, lysine-54 in H^{315}, and lysine-50 in L^{460}. These labeled residues all fall in the restricted hypervariable regions of V_L and V_H; almost certainly, therefore, these regions form the antibody combining sites. A novel bifunctional Dnp-labeling reagent cross-linked L^{315} and H^{315} via tyrosine-34 in the former and lysine-54 in the latter, demonstrating that, in the intact protein, hypervariable regions of V_L and V_H lie within 5A of each other. (Abbreviations: L^{315}, H^{315}, L^{460}, are light (L) or heavy (H) chains from proteins 315 and 460, respectively.)

Immune Responses to Myeloma Proteins and Myeloma Tumors.

BALB/c mice immunized with protein 315 or protein 460 failed to make antibodies to any but the unique, idiotypic determinants of these proteins. These determinants are evidently located in the protein combining sites, because their reactions with the anti-idiotypes were specifically blocked by small Dnp ligands.

BALB/c mice immunized with protein 315 rejected grafts of the corresponding tumor cells (MOPC-315). With large challenging inocula, some immunized mice developed tumor variants that no longer secreted protein 315.

Strange Cross Reaction Between Dnp and Vitamin K_3.

Myeloma proteins with anti-Dnp activity and some conventional anti-Dnp antibodies have substantial affinity for vitamin K_3 (menadione; 2-methyl-1,4-naphthoquinone). To study further the strange cross reaction between these structurally dissimilar haptens, antibodies are being produced to K_3 with an immunogen in which K_3 is coupled through thioether bond to SH groups of homocysteinyl substituents of proteins. Through the same kind of linkage, K_3-specific immunoadsorbants and K_3-haptens have been synthesized.

A. Primary Structure of Variable Domains of Anti-Dnp Myeloma Proteins.

Conventional sequencing technics aided by affinity-labeling of specific residues with a variety of Dnp-bromoacetyl derivatives has yielded the following results.

1. A complete sequence for the light chain of protein 315 (L^{315}) has been derived (214 residues). In this chain the tyrosine residue in position 34 is exclusively and specifically labeled in high yield (75% of theoretical value) when the intact protein binds BADE (bromoacetyl-Dnp ethylenediamine). (Haimovich et al 1970, Schulenburg et al 1971, Haimovich et al. in press).

L^{315} is clearly λ-like. We believe it represents a third type of light chain (which we designate λ2), because its constant region differs considerably (27% of the residues) from that of other mouse λ chains. However, in its variable region, L^{315} has only few differences from other mouse λ's: e.g., L^{315} and L^{104} (from protein 104) differ in only 8 of 110 residues in their respective V_L segments. Yet proteins 104 and 315 differ drastically in specificity: L^{104} is anti-dextran, with no anti-Dnp activity, while 315 is anti-Dnp and has no anti-dextran activity. Either these 8 residues (half of them in hypervariable regions) have an immense effect on specificity of the combining site, or this specificity is largely determined by sequences in V_H (see below).

2. To complete the primary structure of the variable region of protein 315, this protein's heavy (α) chain (H^{315}) is being analyzed. After CNBr digestion, 5 fragments corresponding to the 4 methionine residues were purified, and the N- and C-terminal fragments were identified from their correspondence to the N-terminal and C-terminal sequences of the intact heavy chain.

The C-terminal fragment (9 residues) has been sequenced. The N-terminal fragment consists of 148 residues, and includes the entire variable segment (V_H). After blocking NH_2 groups of its lysine and aminoethylcysteine residues, this fragment was digest with trypsin: 5 peptides, corresponding to the 4 arginine residues, were isolated, aligned, and have been partially sequenced.

Peptide 1 (25 residues) is the N-terminal residue. As it has a free NH_2 group (Aso) it has been possible (in collaboration with Dr. Leroy Hood) to establish its sequence with the automatic Edman sequencer.

Peptide 2 (38 residues) carries the lysine residue that was exclusively and specifically labeled in high yield (80% of theoretical) when the intact protein binds BADL (N^{α}-bromoacetyl-N^{ϵ}-Dnp-L-lysine). This peptide was completely sequenced in collaboration with Dr. David Givol's group (at the Weizmann Institute, Rehovot, Israel). This established definitively that the labeled residue is at position 54.

Peptide 3 (5 residues) has been sequenced by conventional technics.
Peptide 4 (48 residues) has been partially sequenced.
Peptide 5 (32 residues) has been partially sequenced.
All together the sequence for H^{315} has been established for residues 1 to 88.

3. Similar approaches to protein 460 have shown that in its light chain the lysine residue at position 50 is affinity-labeled by BADE (see above). Altogether residues 1-63 of this chain have been sequenced (Haimovich, Hood and Eisen).

In each of the foregoing studies only residues in the restricted hypervariable portions of the V domains of light and heavy chains were specifically derivatized by affinity-labeling reagents. The findings provide strong support for the intuitive supposition that residues of these hypervariable regions form the active combining sites of antibodies.

Moreover, a specially fabricated bifunctional affinity-labeling reagent (a Dnp-bisbromoacetyl derivative of diaminobutyric acid hydrazide) with two bromoacetyl groups, one as far from the Dnp as in BADE and the other as far from the Dnp as in BADL, was bound specifically by protein 315 and covalently cross-linked the tyr-34 of L^{315} and lys-54 of H^{315}, demonstrating that in the intact protein the hypervariable regions of light and heavy chains are within 5A of each other (Givol *et al*, 1971). This provides structural support for the view that both V_L and V_H jointly establish the specific combining site.

B. Immunity to Myeloma Protein's and Myeloma Tumors

1. *Autoimmune-like Antibodies to Myeloma Proteins*. BALB/c mice immunized with protein 315 or protein 460 made antibodies that reacted only with the unique, idiotypic determinants of the respective proteins. These determinants appear to be located in the protein's combining sites, because Dnp ligands specifically block the reaction between these BALB/c immunoglobulins and their respective BALB/c anti-idiotypic antibodies (Sirisinha and Eisen, 1971). The results are in accord with the expectation that an animal should be tolerant of those determinants of his monoclonal immunoglobulins (isotypic and allotypic) that are shared with his normal immunoglobulins; but might be induced to make an immune response to the unique, idiotypic determinants of the monoclonal proteins.

2. *Rejection of Myeloma Tumor Grafts*. If a given myeloma tumor has a myeloma protein appropriately localized in the cell membrane, antibodies or "killer" lymphocytes that react specifically with that myeloma protein might after the proliferation of the tumor cells. This possibility was tested by inoculating MOPC-315 cells into mice that had been immunized with protein 315. With an inoculum (5×10^3 cells) that produced fatal tumors in 90% of unimmunized controls, tumors developed slowly in only 10% of the immunized animals. With a larger inoculum (100% fatal tumors in unimmunized controls), tumors eventually developed in ca. 40% of immunized animals. However, the tumors appeared only after a lag in the immunized animals, *and in all instances they differed from the original tumor in that they no longer secreted protein 315.*

These results show that under certain circumstances of myeloma protein can function as a "tumor-specific transplantation antigen." The results are also reminiscent of "allotype-suppression" and "antigenic modulation", two other phenomena in which antibodies to surface immunoglobulins or other isoantigens of a lymphoid cell eliminate the cell's replicative capacity, or block its synthesis of the surface isoantigen (immunoglobulins or others).

C. The Strange Cross Reaction Between Dnp and Vitamin K_3.

It was found earlier that protein 315 bound menadione (vitamin K_3; 2-methyl-1,4-naphthoquinone) specifically in the same site, or an overlapping one. that bound Dnp ligands. The same cross reaction was then seen with conventional anti-Dnp antibodies. This strange cross reaction is being analyzed further by studying Dnp binding by antibodies to vitamin K_3, as well as K_3 binding by anti-Dnp.

Antibodies to vitamin K_3 can be prepared by immunizing rabbits with K_3-conjugates (e.g., B G with homocyteinyl substituents to whose SH groups K_3 is attached via thioether linkage to C-3 of menadione_, and isolating anti-K_3 from antisera with the aid of Sepharose immunoadsorbants with covalently attached K_3. The same adsorbants separate anti-Dnp populations into subsets; e.g., about 50% of late, high-affinity anti-Dnp antibodies are selectively isolable on K_3-adsorbants. With the recent synthesis of ^{14}C-K_3-homocysteinyl-aminobutyrate as a stable hapten, work is now proceeding on the binding and competitive binding of Dnp and K_3 ligands by selected populations of anti-Dnp and anti-K_3 antibodies. The results may help explain the high frequency of Dnp-reactive myeloma proteins in mice (Johnston and Eisen).

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b. In Press

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APPENDIX - I

Autoimmune-like Antibodies to Idiotypic Determinants in Combining Sites of Myeloma Proteins (ref. 7).

TABLE 1. *Response of inbred mice to BALB/c A-myeloma proteins*

			Percent radioactive antigen precipitated†			
			Second bleeding		Third bleeding	
Group*	Strain	Immunized and tested with	Range	Mean	Range	Mean
1	BALB/cAnN	315	——‡		22–41 (6)	29
2	BALB/cAnN	315	36–47 (10)§	42	47–51 (9)	49
3	BALB/cJ	315	46–50 (10)	47	47–52 (4)	50
4	BALB/cJ	315	6–25 (8)	14	19–48 (7)	28
5	C57BL/10Sn	315	36–51 (6)	46	36–48 (8)	42
6	C3H/Sn	315	45–52 (7)	49	47–54 (8)	52
7	A/J	315	46–53 (10)	49	——	——
8	BALB/cJ¶	BrAc-Dnp-Lys-315	0 (4)	0	——	——
9	C57BL/10Sn¶	BrAc-Dnp-Lys-315	5–39 (3)	18	——	——
10	BALB/cJ	460	——		——	24 (4)
11	C57BL/10Sn	460	——		19–25 (8)	22
12	BALB/cAnN	22A	0–7 (9)	0	0‖ (4)	0

---

* Mice in group 1 received the first and second injections of immunogen as alum-precipitated protein. In group 4, the first two injections were intraperitoneal. For all other ~~details~~ see *Methods*.

† ~~This~~ testing for antibody activity, 25 μl of antiserum was incubated (37°C, 30 min) with an excess of ~~labeled~~ [^{125}I] antigen ~~([^{125}I]315, [^{125}I]460, [^{125}I]22A or [^{125}I]Dnp-HSA)~~ in 375 μl of PBS-BSA. After addition of 100 μl of goat anti-mouse IgG (and incubation at 37°C, 60 min, then at 4°C, 18 hr), immune precipitates were washed and counted. Numbers are percent of added radioactivity recovered in the precipitate after correction for blanks prepared with normal mouse sera. Blanks varied from 1–4% of added [^{125}I]antigen, except for [^{125}I]460 (see *text*). Experimental values that differed from the blanks by less than twice the standard deviation of the blank are represented as zero.

Some mice with high titers in second and third bleeding had no detectable antibody in their first bleeding. ~~Average titers usually rose between the first and second bleeding.~~

‡ ——, not tested.

§ Values in parenthesis represent number of mice bled; serum from group 10 was tested only as a pool because of high blanks (see text).

¶ Sera from one of four BALB/c precipitated 21% and two of three C57BL/10Sn precipitated 9 and 34% of added [^{125}I]Dnp-HSA.

‖ One of these four survivors formed anti-protein 22A antibody after further immunization (8% of [^{125}I]22A precipitated versus 4% for the blank); thus two of nine BALB/c mice ~~appeared to give~~ have given a feeble response to 22A.

TABLE 2. *Specificity of mouse antisera to BALB/c A-myeloma proteins*

Strain	Immunized with	Percent of radioactive antigen precipitated*				Inhibition† of precipitation of [^{125}I]315 by	
		Protein-315	BrAc-Dn-Lys-315	460	22A	Protein 315 (μg for 50% inhibition)	BrAc-Dnp-Lys-315 (% inhibition by 0.85 μg)
BALB/cAnN	315	49	——‡	0	0	——	——
BALB/cJ	315	28	0	0	0	0.47	0
C57BL/10Sn	315	42	——	0	0	0.44	13
C3H/Sn	315	52	——	23	0	——	——
A/J	315	49	——	21	0	——	——
BALB/cJ	BrAc-Dnp-Lys-315	0	——	——	——	——	——
C57BL/10Sn	BrAc-Dnp-Lys-315	39	39	——	0	——	——
BALB/cJ	460	0	——	24	0	——	——
C57BL/10Sn	460	5	——	22	0	——	——
BALB/cAnN	22A	0	——	——	7	——	——

* All antigens were labeled with ^{125}I, except BrAc-Dnp-Lys-315, which contained the ^{14}C affinity label; see legend to Table 1 for details of the assay, and definition of zero.

† Either Protein-315 or BrAc-Dnp-Lys-315 was preincubated (25°C, 30 min) with 10 μl of anti-Protein-315 before 0.08 μg[^{125}I]315 was added. Thereafter, the reaction mixtures were treated as described in Table 1.

‡ ——, not tested.

Only mea
values
listed.

From ref. 7

APPENDIX - II

Tentative Sequence of Light Chain of Protein 315 (from ref. 6).

TENTATIVE SEQUENCE OF L-315

LIGHT CHAIN OF THE IgA FROM MOPC-315

1 PCA ALA/VAL VAL THR GLX GLX SER ALA LEU THR THR SER PRO GLY GLY THR/THR VAL/LEU

21 THR $\boxed{\text{CYS}}$ ARG)SER SER THR GLY ALA VAL THR THR SER ASN $\overset{*}{\text{TYR}}$ ALA ASN TRP ILE GLX GLX

41 PRO(ASX LYS HIS LEU PHE THR GLY)LEU(ILE GLY GLY THR SER ASX)ARG ALA PRO GLY VAL

61 PRO VAL ARG/PHE SER GLY SER(LEU ILE GLY ASX)LYS/ALA ALA/LEU THR/ILE THR GLY(ALA

81 GLX THR GLX ASX GLX ALA(MET)TYR PHE $\boxed{\text{CYS}}$ ALA LEU TRP PHE ARG/ASX HIS PHE VAL PHE

101 GLY GLY GLY THR LYS/VAL(THR VAL LEU GLY GLN PRO)LYS SER THR PRO THR LEU THR VAL

121 PHE PRO PRO SER SER GLX GLX LEU LYS GLU ASN LYS/ALA THR LEU VAL $\boxed{\text{CYS}}$ LEU ILE SER

141 ASX PHE(SER PRO GLY SER VAL THR)VAL ALA TRP LYS/ALA ASX(GLY THR PRO)ILE THR GLX

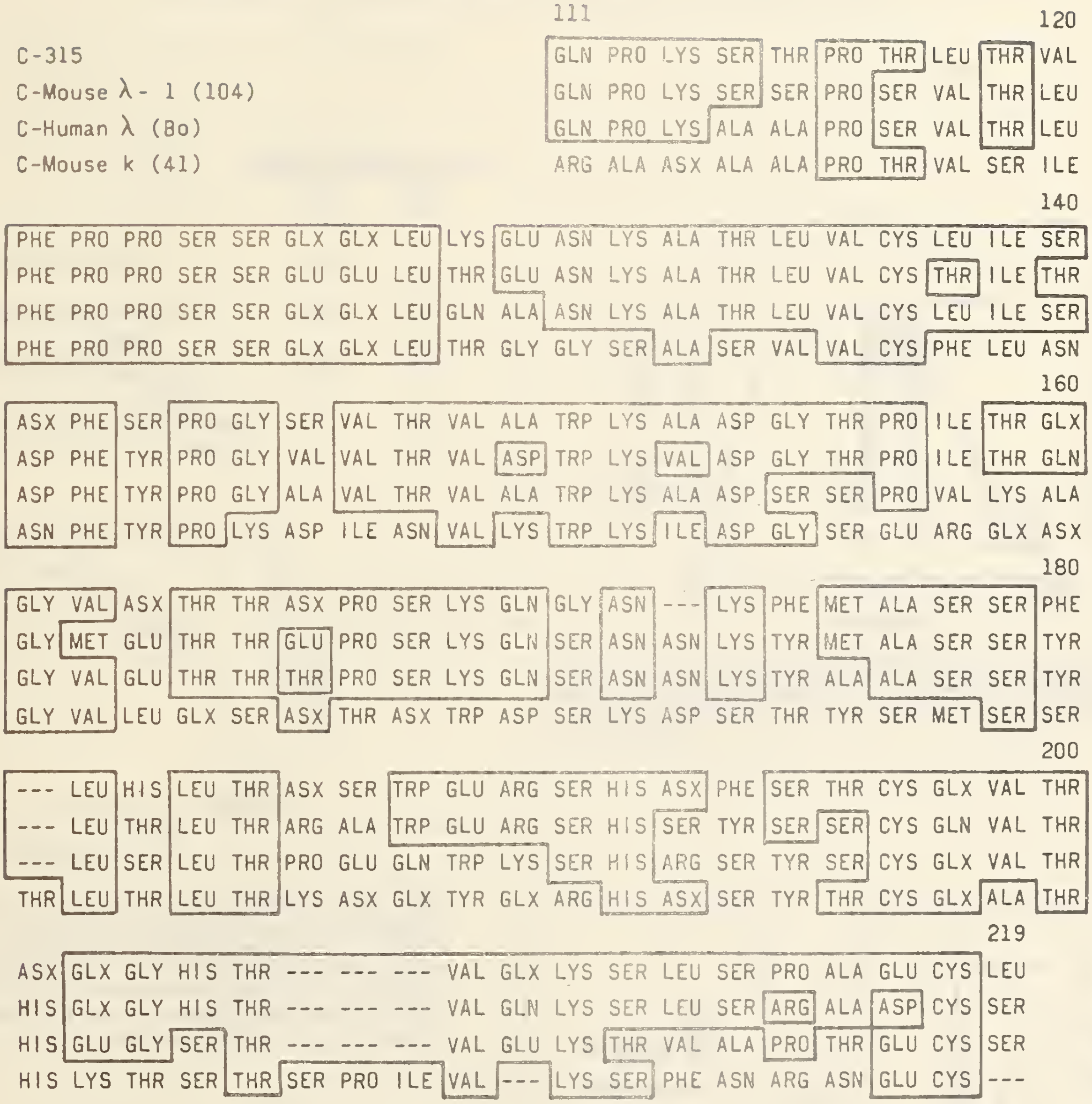
161 GLY(VAL ASX THR THR ASX PRO SER)LYS GLX GLY ASX LYS/PHE MET ALA SER SER(PHE LEU

181 HIS)LEU THR ASX(SER TRP GLX)ARG/SER HIS ASX PHE SER THR $\boxed{\text{CYS}}$ GLN VAL THR ASX(GLX

201 GLY HIS THR)VAL GLX LYS SER LEU SER PRO ALA GLU $\boxed{\text{CYS}}$ LEU

FIG. 1. The tentative amino acid sequence of L^{315}, the light chain of the A-myeloma protein produced by MOPC-315. / indicates that overlaps between peptides have not been established. Residues within parenthesis have been determined by composition and are aligned by homology to the sequence of L^{104} (12). * The tyrosine at position 34 has been labeled specifically with two different affinity-labeling reagents (15, 17). The two methionines and five cysteine residues are enclosed by circles and boxes.

APPENDIX II - cont'd



	111									120
C-315	GLN	PRO	LYS	SER	THR	PRO	THR	LEU	THR	VAL
C-Mouse λ- 1 (104)	GLN	PRO	LYS	SER	SER	PRO	SER	VAL	THR	LEU
C-Human λ (Bo)	GLN	PRO	LYS	ALA	ALA	PRO	SER	VAL	THR	LEU
C-Mouse k (41)	ARG	ALA	ASX	ALA	ALA	PRO	THR	VAL	SER	ILE

																			140
PHE	PRO	PRO	SER	SER	GLX	GLX	LEU	LYS	GLU	ASN	LYS	ALA	THR	LEU	VAL	CYS	LEU	ILE	SER
PHE	PRO	PRO	SER	SER	GLU	GLU	LEU	THR	GLU	ASN	LYS	ALA	THR	LEU	VAL	CYS	THR	ILE	THR
PHE	PRO	PRO	SER	SER	GLX	GLX	LEU	GLN	ALA	ASN	LYS	ALA	THR	LEU	VAL	CYS	LEU	ILE	SER
PHE	PRO	PRO	SER	SER	GLX	GLX	LEU	THR	GLY	GLY	SER	ALA	SER	VAL	VAL	CYS	PHE	LEU	ASN

																			160
ASX	PHE	SER	PRO	GLY	SER	VAL	THR	VAL	ALA	TRP	LYS	ALA	ASP	GLY	THR	PRO	ILE	THR	GLX
ASP	PHE	TYR	PRO	GLY	VAL	VAL	THR	VAL	ASP	TRP	LYS	VAL	ASP	GLY	THR	PRO	ILE	THR	GLN
ASP	PHE	TYR	PRO	GLY	ALA	VAL	THR	VAL	ALA	TRP	LYS	ALA	ASP	SER	SER	PRO	VAL	LYS	ALA
ASN	PHE	TYR	PRO	LYS	ASP	ILE	ASN	VAL	LYS	TRP	LYS	ILE	ASP	GLY	SER	GLU	ARG	GLX	ASX

																			180
GLY	VAL	ASX	THR	THR	ASX	PRO	SER	LYS	GLN	GLY	ASN	---	LYS	PHE	MET	ALA	SER	SER	PHE
GLY	MET	GLU	THR	THR	GLU	PRO	SER	LYS	GLN	SER	ASN	ASN	LYS	TYR	MET	ALA	SER	SER	TYR
GLY	VAL	GLU	THR	THR	THR	PRO	SER	LYS	GLN	SER	ASN	ASN	LYS	TYR	ALA	ALA	SER	SER	TYR
GLY	VAL	LEU	GLX	SER	ASX	THR	ASX	TRP	ASP	SER	LYS	ASP	SER	THR	TYR	SER	MET	SER	SER

																			200
---	LEU	HIS	LEU	THR	ASX	SER	TRP	GLU	ARG	SER	HIS	ASX	PHE	SER	THR	CYS	GLX	VAL	THR
---	LEU	THR	LEU	THR	ARG	ALA	TRP	GLU	ARG	SER	HIS	SER	TYR	SER	SER	CYS	GLN	VAL	THR
---	LEU	SER	LEU	THR	PRO	GLU	GLN	TRP	LYS	SER	HIS	ARG	SER	TYR	SER	CYS	GLX	VAL	THR
THR	LEU	THR	LEU	THR	LYS	ASX	GLX	TYR	GLX	ARG	HIS	ASX	SER	TYR	THR	CYS	GLX	ALA	THR

																		219
ASX	GLX	GLY	HIS	THR	---	---	---	VAL	GLX	LYS	SER	LEU	SER	PRO	ALA	GLU	CYS	LEU
HIS	GLX	GLY	HIS	THR	---	---	---	VAL	GLN	LYS	SER	LEU	SER	ARG	ALA	ASP	CYS	SER
HIS	GLU	GLY	SER	THR	---	---	---	VAL	GLU	LYS	THR	VAL	ALA	PRO	THR	GLU	CYS	SER
HIS	LYS	THR	SER	THR	SER	PRO	ILE	VAL	---	LYS	SER	PHE	ASN	ARG	ASN	GLU	CYS	---

FIG. 2. Comparison of constant regions of light chains of 315, mouse λ (104), human λ (Bo), and mouse *k* (41) (refs. 12, 39, and 18, respectively). Positions at which L^{315} is identical to one or more of the other chains are enclosed. Position numbers are based on Glu at 111 in L^{104} and L^{315}. Gaps are introduced to achieve maximum homology.

From ref. 6.

APPENDIX III

Affinity-labeling and Cross-Linking of Heavy and Light Chains of Protein 315 (Ref. 2).

Reagent		Labeled residue and chain	
		%Tyr (L)	%Lys (H)
BADE	DNP-NH-CH$_2$-CH$_2$-NH-X	96	4
BADB	$\text{DNP-NH-CH}_2\text{-CH}_2\text{-}\underset{\text{COOH}}{\text{CH}}\text{-NH-X}$	87	13
BADO	$\text{DNP-NH-CH}_2\text{-CH}_2\text{-CH}_2\text{-}\underset{\text{COOH}}{\text{CH}}\text{-NH-X}$	66	34
BADL	$\text{DNP-NH-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-}\underset{\text{COOH}}{\text{CH}}\text{-NH-X}$	5	95

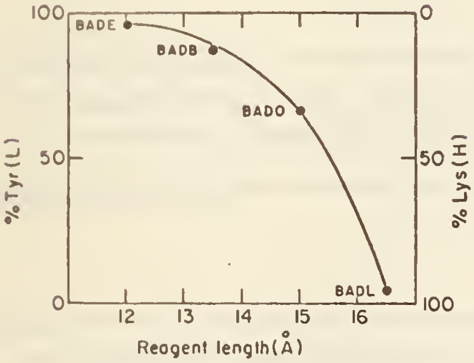


FIGURE 5: The specificity of affinity labeling of protein-315 by different reagents. The upper part of the figure gives the proportion of peptide chain and amino acid residue labeled by the different reagents. This was determined from heavy and light chains isolated on sodium dodecyl sulfate polyacrylamide gels (see Materials and Methods). The lower part gives a schematic representation of the relation between the above specificity and the size of reagents. X = COCH$_2$Br.

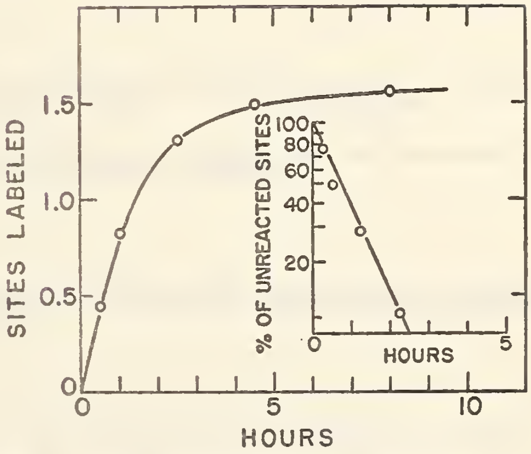


FIGURE 6: Kinetics of labeling of protein-315 by the bifunctional reagent DIBAB. Conditions are as in Figure 1. Insert: replotting the data to indicate the apparent first-order rate constant of reaction as in Figure 2.

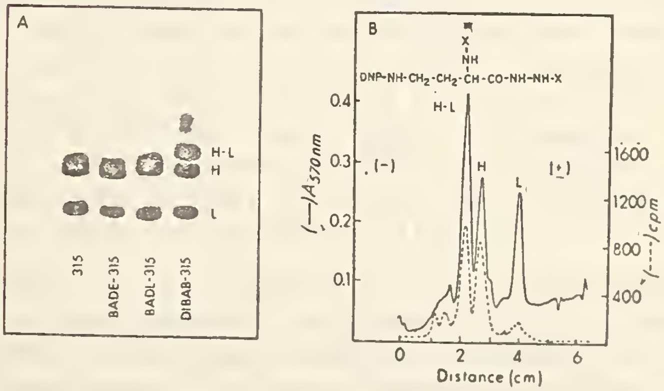


FIGURE 7: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of DIBAB-, BADL-, and BADE-labeled protein-315. The conditions for labeling and sodium dodecyl sulfate acrylamide electrophoresis are given in the text. Protein (100 μg) in 100 μl was applied to the gel. After the run, the gel was stained, and either scanned at 570 nm or sliced to 50 slices (1.3 mm) which were dissolved in Soluene and counted in toluene scintillant. (A) Electrophoretic pattern of affinity-labeled protein-315, protein migration from top to bottom. (B) Distribution of protein and radioactivity in the gel of DIBAB-labeled protein. H, heavy chain; L, light chain. X = COCH$_2$Br.

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13. ABSTRACT

Two mouse myeloma proteins (nos. 315 and 460) that bind Dnp antigens specifically and with high affinity were analyzed with respect to amino acid sequences in variable (V) regions of their light (L) and heavy (H) chains, and the amino acid residues that make-up their specific combining sites. The L chain of protein 315 (L^{315}) has been completely sequenced (residues 1 to 214). Residues 1 to 88 of H^{315} and 1 to 63 of L^{460} have also been determined. Affinity-labeling with various Dnp-bromoacetyl reagents labeled Tyrosine-34 of L^{315}, Lysine-54 of H^{315}, and Lysine-50 of L^{460}. The labeled residue in each case was situated in a hypervariable segment (of the V_L or V_H region); these small segments seem therefore to form the specific combining sites.

BALB/c mice immunized with purified protein 315 made antibodies that reacted specifically with unique determinants (idiotypes) in the protein's Dnp-combining site. The immunized mice also rejected grafts of MOPC-315, the myeloma tumor that produces protein 315; this protein can thus behave as a "tumor-specific transplantation antigen."

To study the strange cross-reaction between Dnp and Vitamin K_3 (2-methyl-1,4-naphthoquinone), antibodies to K_3 were raised by immunizing rabbits with K_3-protein conjugates in which K_3 was covalently attached to protein SH groups via thioether linkage. The same type of reaction was used to prepare K_3-specific solid immunoadsorbants and various K_3-haptens.

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REPORT NUMBER 4

CLASSES AND PROPERTIES OF HUMAN ANTIBODIES

ANNUAL REPORT

DR. BERNARD B. LEVINE

1 June 1970 - 31 May 1971

Supported by

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Office of the Surgeon General, Washington, DC 20314
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of the Armed Forces Epidemiological Board

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SUMMARY

The following studies were carried out:

1. Effect of time interval between antigen doses on immune responses in the mouse.

Decreasing the interval between injections of antigen increased IgG and decreased reagin antibody titers. This pertains to immuno-therapy of human allergy.

2. The production of hapten-specific reaginic antibodies in the guinea pig.

Reaginic antibodies in high titers were raised in guinea pigs by the alumina gel-low dose antigen method. Reagin had properties similar to IgE in man.

3. Genetic control of reaginic antibody responsiveness in mice.

SJL mice make little or no serum reagin while making good IgG responses, whereas RF make both. Differences are under genetic control by more than one autosomal locus.

4. H-2 linked control of immune responsiveness in mice.

Genetic experiments indicate control by a single autosomal locus closely linked to the H-2 system.

FOREWORD

We wish to acknowledge the competent technical assistance of Mrs. Louise Pfister.

In last year's report we recorded our studies showing the development of systems of reagin production in mice as models for studying mechanisms and controls of reagin production in human atopic diseases. We showed methods of inducing persistent high titered reagin production in mice to low doses of protein antigens, the effect of antigen, antigen dose and mouse strain on immune responsiveness and reagin production and its correlation with H-2 genotype. This year we report genetic studies on the low dose - H-2 correlated system, further operational studies on immunization, extension of the low-dosage system to guinea pigs and the demonstration of a reaginic homocytotropic antibody in the guinea pig, and a new kind of genetic control of reagin production in mice.

The following studies were carried out:

1. Effect of time interval between antigen doses on immune responses in the mouse (B.B. Levine and H. Chang, Jr.) Immunization with repeated minute doses of antigen was used and the effect of varying the interval between doses was studied. A/He mice were used and benzylpenicilloyl-bovine γ-globulin ($BPO_{25}BGG$) in alumina gel given i.p. was the antigen. A grid type of immunization protocol was set up; doses varied from 0.01 to 1.0 μg antigen; booster intervals varied from one to 4 weeks. Reagin and γ_1 antibodies were assayed by PCA in CFW mice using 2 hour and 48 hour sensitization periods. The validity of the assay is good and was described in last year's report. The results are shown in Table 1. The notable finding was an effect of time interval between antigen injections to dissociate IgG_1 from reaginic antibody production. For example, in the groups given 1.0 μg doses, the ratios of titers IgG_1/reagin were about 65:1 for the one week interval and 8:1 to 1:1 for the 4 week interval. Going from 4 to one week, IgG titers increased 4 to 8-fold and reagin titers decreased 2 to 4-fold. The reasons for these findings are not clear. One possibility is that the primary effect of frequent antigen injections may be to increase IgG_1 antibody production and that IgG antibody may specifically suppress reagin production. These observations pertain to production of high titered serum reagin in mice. They also serve to raise the question of the importance of time interval in determining the therapeutic results of immuno-therapy in atopic allergy.

2. The production of hapten-specific reaginic antibodies in the guinea pig. (B.B. Levine, H. Chang, Jr., and N.M. Vaz) Homocytotropic (HCT) antibodies of the IgE type (reagin) as well as γ_1 HCT antibodies were produced in the guinea pig by immunization with repeated 1.0 μg doses of $BPO_{25}BGG$ mixed with 1.0 mg alumina gel injected i.p. This seems to be the first demonstration of reagin in the guinea pig produced to non-living antigens. Both hapten (BPO) and protein-specific reaginic antibodies were produced. Titers of HCT antibodies were assayed by PCA in guinea pigs, γ_1 antibodies with a latent period of 4 hours, reaginic antibodies with a latent period of 192 hours (8 days). Titers as high as 1:4000 were obtained for both γ_1 and reagin antibodies. γ_1 and reagin were distinguished by the following properties: γ_1 was absorbed by a monospecific rabbit anti-guinea pig γ_1 serum; reagin was not. γ_1 was not heat labile (56°C x 2 hours); reagin was heat labile. As assayed, reagin persisted in skin for considerably longer time periods than did γ_1 antibodies. Our data also suggest

that approximately 5% of the 4 hour titer persists after 8 days, and that approximately 5% of the 8 day reagin titer can be already detected after a 4 hour sensitization period. However, in the standard PCA assay, these "contaminents" are negligible and usually insignificant. This "low dose-alumina gel" method of production of reagin has also worked well for mouse (Levine, B.B. and Vaz, N.M., Int. Arch.Allergy, 39:156-71,1970) and appears to be working well for rabbits. It appears to be a useful general method for the production of reagin to non-living antigens. These systems show promise as experimental models for human allergy permitting studies aimed at development of more rational therapies than now exist.

3. Genetic control of serum reaginic antibody responsiveness in mice (B.B. Levine and N.M. Vaz) In many other studies on genetic controls of immune responses, control was upon responsiveness in general, and reagin production was only a part of the more general responsiveness. In these present experiments we have observed a kind of genetic control which is upon serum reagin responsiveness uniquely. Numerous immunization experiments with the SJL and RF strains showed that whereas both produce γ_1 antibodies in high titer, RF mice are capable of producing reagin in high titer whereas the SJL produce only trace serum titers of reagin in undetectible amounts. Some sample data with $DNP_{42}BGG$ are given in Fig.1. This same difference between RF and SJL was observed with 7 other protein and hapten-protein antigens. No effect of antigen dose was seen, i.e., for ovalbumin, between doses of 0.1 to 100 μg the same difference in serum reagin production was seen. Thus we have no evidence for a "split tolerance" effect involving reagin. Nor was there evidence for a possible "negative feedback" from IgG antibody accounting got this difference. Further, we found no evidence for reagin of strain-specific restriction in skin-binding avidity, i.e., SJL sera were negative for reagin in SJL recipients as well as in other strains. Finally, we studied the possibility that reagin might be cleared from the serum much faster from SJL than from RF mice. Passive transfers of high titered reaginic sera were done into RF and SJL and the fall off in reaginic titers were not strikingly different (Fig. 2). Thus our provisional interpretation is that the RF and SJL differences are due mainly to differences in reagin production.

Genetic controls were studied by breeding experiments with the various generations immunized to test for reagin production. The data are shown in Fig. 3. Peak reagin titers are listed for individual animals. Peak γ_1 antibody titers are not shown but were roughly equal for the generations. The F_1 generations were all reagin producers, i.e., the trait was dominant. In the backcross, there was no clear distribution. If one segregates into groups with limits of the parental strains, 3 groups emerge: good producers like RF, poor producers like SJL, and intermediate serum producers not represented in the parental strain. The validity of the intermediate group was studied by crossing them to SJL. Of the offspring, 8 were poor producers and 8 were intermediate. There were no good producers. These data indicate genetic control by more than one locus. The distribution, not significantly different from 50:25:25, is consistent with 2 loci, but this interpretation will require work with more animals. This system, where control is upon a unique antibody class, reagin, and where no antigenic specificity was seen, differs from the H-2 associated system, described below, where control is upon responsiveness and shows distinct antigenic specificity. The data thus show at least two different genetic controls of reagin production in the mouse, having different immunological and genetic properties.

4. H-2 linked genetic control of immune responsiveness to ovalbumin and ovomucoid (N.M. Vaz, J.M. Phillips-Quagliata, B.B. Levine and E.M. Vaz) As reported in last year's annual report, low dose responsiveness to ovalbumin and ovomucoid occur in strains of different H-2 genotype. In the present studies breeding experiments were carried out to determine the genetic transmission of responsiveness and its linkage to the H-2 system. The following findings were made: 1) F_1 generations between responders and non-responders were responders, i.e., the trait is dominant. 2) The F_1 x non-responder backcross yielded 50% responders indicating control by a single locus. 3) There was no cross-over with H-2 indicating close linkage. 4) Congenic strain pairs responded as predicted by their H-2 indicating again close linkage. In these experiments both PCA and radio-immunoprecipitation assay techniques were used with similar results.

Table I. Effect of antigen doses and time interval between doses upon BPO-specific IgG_1 and reagin antibody titers[1]

Primary dose µg	Booster dose µg	Booster frequency[2] week	Antibody titers at[3] 5 weeks		9 weeks		13 weeks	
			Reagin	IgG_1	Reagin	IgG_1	Reagin	IgG_1
0.10	0.01	1	5	5	5	40	5	160
0.10	0.01	2	0	0	0	0	0	0
0.10	0.01	3	0	0	0	0	0	0
0.10	0.01	4	0	0	5	5	5	10
0.10	0.10	1	20	80	20	640	10	1,280
0.10	0.10	2	20	40	20	80	20	320
0.10	0.10	3	5	5	40	40	20	160
0.10	0.10	4	5	5	40	80	20	160
1.0	0.01	1	5	40	0	80	0	640
1.0	0.01	2	5	20	5	160	0	320
1.0	0.01	3	20	40	40	80	5	80
1.0	0.01	4	20	40	40	80	20	160
1.0	0.10	1	20	320	20	1,280	20	2,560
1.0	0.10	2	40	160	40	640	20	640
1.0	0.10	3	40	80	40	160	40	320
1.0	0.10	4	40	80	80	320	40	320
1.0	1.0	1	20	1,280	40	2,560	40	2,560
1.0	1.0	2	40	320	20	640	20	1,280
1.0	1.0	3	160	160	80	320	160	640
1.0	1.0	4	320	320	80	640	160	640

[1] $BPO_{25}BGG$ plus 1.0 mg Al $(OH)_3$ gel l.p. in A/He mice.

[2] Antigen injections were given at intervals of 1, 2, 3 or 4 weeks.

[3] Bleedings were always 1 week after the last antigen injection. The 3 week group was actually bled at 4 weeks, 7 weeks, 10 weeks and 13 weeks. The 4, 7 and 13 weeks titers are given.

Fig. 1

IMMUNE RESPONSE OF INDIVIDUAL RF AND SJL MICE TO DNP$_{42}$-BGG

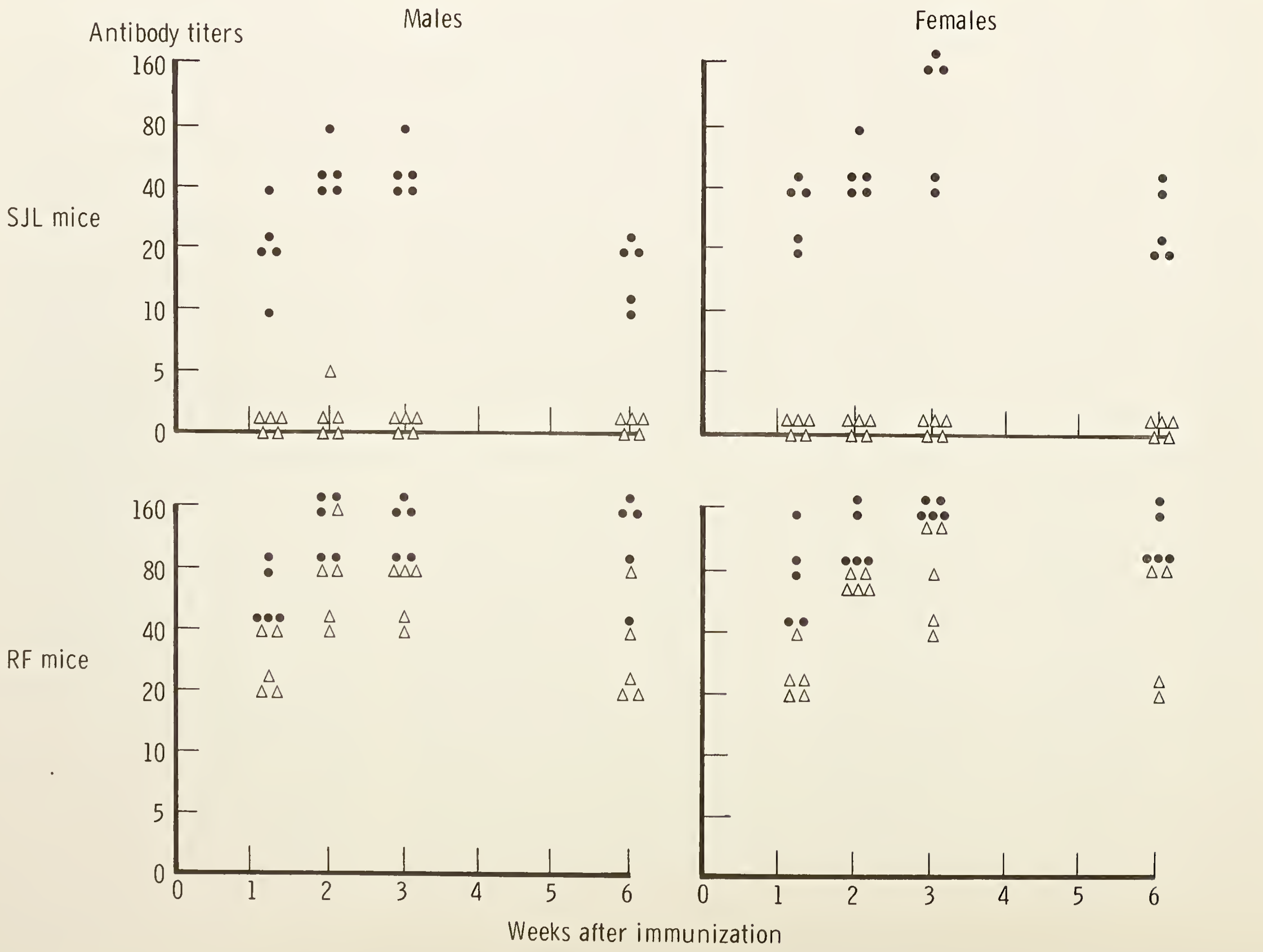


Fig. 2

CLEARANCE OF REAGIN FROM THE SERUM OF RF (○) AND SJL (●) MICE

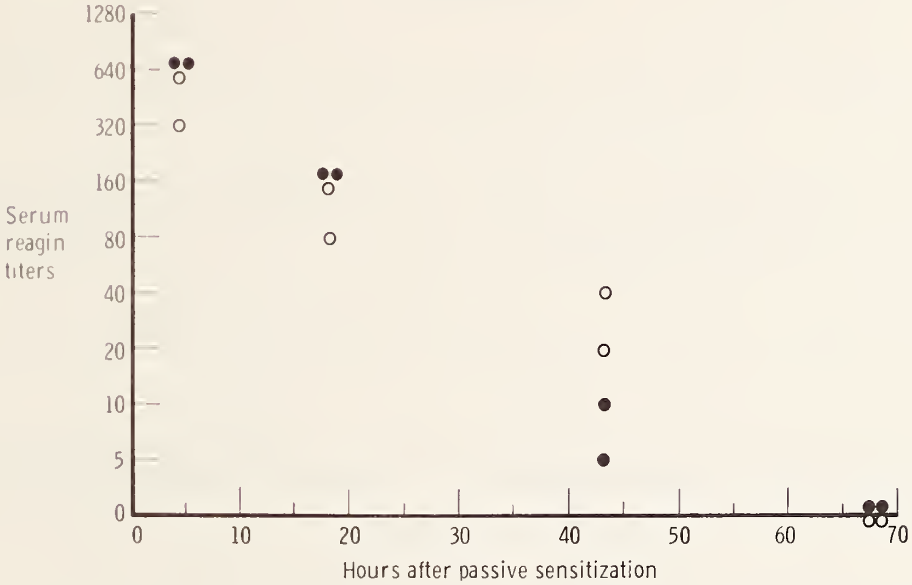
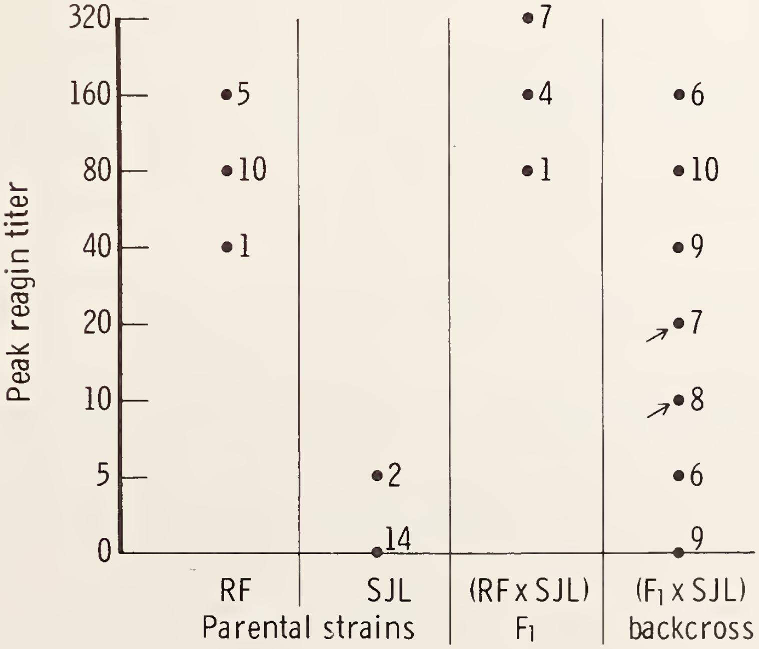


Fig. 3

REAGINIC RESPONSES TO ONE LARGE DOSE OF $DNP_{35}BGG$



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2. Levine, B.B. Genetic factors in reagin production in mice. Second International Symposium on the Biochemistry of the Acute Allergic Reaction, 1971.

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13. ABSTRACT

The following studies were carried out:

1. Effect of time interval between antigen doses on immune responses in the mouse. Decreasing the interval between injections of antigen increased IgG and decreased reagin antibody titers. This pertains to immuno-therapy of human allergy.

2. The production of hapten-specific reaginic antibodies in the guinea pig. Reaginic antibodies in high titers were raised in guinea pigs by the alumina gel-low dose antigen method. Reagin had properties similar to IgE in man.

3. Genetic control of reaginic antibody responsiveness in mice. SJL mice make little or no serum reagin while making good IgG responses, whereas RF make both. Differences are under genetic control by more than one autosomal locus.

4. H-2 linked control of immune responsiveness in mice. Genetic experiments indicate control by a single autosomal locus closely linked to the H-2 system.

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ATOPY						
HISTOCOMPATABILITY ANTIGENS						
H-2						
MOUSE IMMUNOGLOBULINS						
ANTIGENS						
GUINEA PIG ANTIBODIES						
SJL MICE						

AD______________

ISOLATION AND CHARACTERIZATION OF LYMPHOGRANULOMA VENEREUM AGENTS (U)

ANNUAL PROGRESS REPORT NO. 2

by

K. F. Meyer, M.D.

September 1971
(For the period 1 June 1970 to 31 May 1971)

Supported by

U.S. ARMY MEDICAL RESEARCH & DEVELOPMENT COMMAND
Office of The Surgeon General, Washington, D.C. 20314
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of the Armed Forces Epidemiological Board

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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

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SUMMARY

Only 6 new lymphogranuloma venereum (LGV) cases were seen this year. The reduced number may reflect an actual decrease or may indicate fewer referrals to the San Francisco hospitals.

Experimental Frei test antigens were tested in LGV-infected rabbits and monkeys. The commercial Lygranum antigen, which has been a relatively poor diagnostic tool in humans, was included. It was also a poor skin test antigen in the experimental system. This system was far from ideal, but positive tests of delayed hypersensitivity were obtained with a number of experimental antigens prepared from recently isolated LGV strains. It is suggested that development of an improved antigen, possibly including recent isolates, be undertaken.

Complement fixation (CF) tests, performed on the sera of 92 servicemen spending at least a year in Vietnam, yielded a 6% rate of positives. This rate is approximately twice the rate in a young male civilian population but the size of the sample is too small to draw any conclusions. Studies in progress, of experimental LGV infections and methods for serotyping LGV isolates, are described.

Serologic response in experimental LGV infection has been studied. The time sequence of specific immunoglobulins has been studied. Anomalous results obtained with whole serum and fractions indicate the possible presence of inhibitors of CF in whole serum. This may be significant in the use of CF as a diagnostic test.

FOREWORD

The authors acknowledge the collaboration of Major Vern Juchau, Medical Service Corps, whose studies on serologic response to lymphogranuloma venereum experiments are included in part in this report. These studies, performed in this laboratory, are in partial fulfillment of the requirement for the Ph.D. degree and will be reported more completely to the Surgeon General's Office.

The technical assistance of Joyce Banks, Fran Clever, Minnie Sung, and Nancy Sugg is appreciated.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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1. Isolation attempts.

During the past year, 6 patients diagnosed as having lymphogranuloma venereum (LGV) were studied in this laboratory. No chlamydial isolates were obtained from these patients. Diagnosis in 4 of these cases was proved on the basis of positive Frei and complement fixation (CF) tests. Inability to recover chlamydiae (bedsoniae) from any of the patients almost certainly reflected their previous exposure to chemotherapeutic agents. We have not had an opportunity to study any fresh, untreated cases.

The decline in the number of patients with this disease seen at this center (including those referred to us from Letterman General Hospital and from the U.S. Public Health Service Hospital in San Francisco, where we collaborate respectively with Col. J. J. Deller, Jr. and Doctor Axel Hoke) probably reflects actual diminution in the number of cases seen. In addition, it may, in part, reflect increased use of antibiotics in the area where the original infections have been contracted. We also collaborate with local venereal disease clinics. Of considerable significance is the fact that we have not seen any major increase in the number of LGV cases in our local civilian population. Most cases in this area have been directly traceable to exposure in Southeast Asia. Two proven clinical cases in civilians were in contacts of returning servicemen. Fortunately, the concern that such servicemen and merchant seamen might import fresh strains into the United States, when they enter as carriers of the agent or while the disease is still in its incubation period, apparently has not been warranted.

At present we are trying to find out whether it is possible to have all patients diagnosed in Vietnam as having LGV evacuated to Letterman Hospital. If feasible, it will increase the number of cases available for study here in the coming year. Most of such incoming evacuees would have received chemotherapeutic agents while in Vietnam, which, of course, would be continued en route, and negate isolation attempts. However, we would still be able to carry out certain laboratory tests and evaluate the therapeutic efficacy of tetracycline and sulfa drugs.

As stated in previous progress reports, all isolates obtained in this study have been highly sensitive in the laboratory to tetracycline and sulfonamides. Their marked laboratory sensitivity contrasts with the erratic and irregular results obtained in treatment of the human disease.

II. Evaluation of the Frei test in laboratory animals.

Because studies on patients with LGV indicate that the Frei test is a relatively insensitive diagnostic tool, attempts have been made to evaluate skin-test antigens prepared from recently isolated strains. The rationale for this has been to determine whether slight modifications in preparation of these antigens or changes in the isolate used to prepare the antigen might increase the sensitivity of the test. Such tests have all been performed on laboratory animals as no preparation is deemed usable on an experimental basis in man.

Antigens tested are of 2 basic types: the Barwell[1] type (acid extract) antigens, developed after the commercial Lygranum antigen was available and claimed to be superior in sensitivity and specificity to the crude yolk-sac preparation in commercial use, and the Lygranum type antigen.[2] The latter is simply prepared by slight purification (by differential centrifugation of infected yolk sacs or tissue culture material and then inactivating the agent with the addition of formaldehyde and phenol). The acid-extract type of antigen is made by partially purifying the elementary bodies and treating them with 0.1 normal HCl for 30 minutes, neutralizing and spinning the particles out. The soluble supernatant material is used as the antigen. Both antigens are prepared from starting material of infected yolk sac or infected tissue cultures similar in numbers of elementary body particles and egg-infectious units. Specific protocols are presented in Appendix I. Nine separate isolates were used to prepare antigens, and all four types (2 types from 2 different host systems) were prepared from each strain. Suitable uninfected control antigens were prepared from each host system used for propagation of agent. A commercial Lygranum* (use of this product does not constitute endorsement) antigen and control, in date, was also used in each run.

The infected animals used as test subjects received intravenous or intramuscular inoculation of yolk-sac-grown agent. The same animals were also used for serologic studies, and in some experiments a series of animals receiving graded doses of agent were used. The serologic response in all animals was significant, whether by fluorescent antibody (FA) methods (for monkeys and rabbits) or by CF test (monkeys only).

---

[1] Barwell, C. F.: Some observations on the antigenic structure of psittacosis and lymphogranuloma venereum viruses. II. Treatment of virus suspensions by various reagents and the specific activity of acid extracts. Brit. J. Exp. Path., 33:268-279, 1952.

[2] Grace, A. W., Rake, G., and Shaffer, M. F.: New material (Lygranum) for performance of Frei test on lymphogranuloma venereum. Proc. Soc. Exp. Biol. Med., 45:249-263, 1940.

*Lymphogranuloma Venereum Skin Test Antigen, Lederle Laboratories

A total of 27 rabbits and 16 monkeys were skin-tested in these experiments. Normal (uninfected) animals did not react to the antigens. The animals were tested by shaving the sides of rabbits or the chests and abdominal areas of the monkeys and inoculating 0.1 cc of test antigen intradermally. The same batches of antigens were tested over a 6-month period. The acid extract antigens were generally nonreactive, offered no advantage in either specificity or sensitivity, and they will not be discussed further. With the cruder elementary body suspension antigens of the Lygranum type considerable erythematous reaction with normal yolk-sac components was found in most rabbits tested, but this was not a problem in monkeys. Only papules were considered to be true delayed hypersensitivity reactions. The tests were read at 48, 72, and 120 hours, with maximal positive results at 48 hours; and a papule about 9 mm in diameter was the maximum reaction obtained. Erythematous zones were not considered meaningful. All papules were measured and any more than 4 mm in absence of control reactions were considered positive. Antigens grown in either tissue culture or yolk sac seemed to give approximately the same number of positive reactions. One advantage in the tissue culture material was that yolk-sac reactivity was less. Control antigens prepared from tissue culture were grossly cleaner and caused no reactions. One strain did not appear to be appreciably superior to another although in some runs homologous systems gave more positives. Results obtained in these titrations were erratic. Some animals did not give any positive tests. The system used was not highly reproducible, as the response from animal to animal varied considerably. One conspicuous finding was that the commercial Lygranum antigen did not react specifically in the test system. Tables 1-4 summarize some representative results.

Firm conclusions cannot be drawn from experiments such as these. The experimental host system presented difficulties and infection in animals used did not really parallel human disease. It is quite likely that the granulomatous response and lymph node involvement in man may be important in developing delayed hypersensitivity reactions. However, some useful information has emerged, which can be summarized by stating that in all systems tested whether related to animal infection or human disease, the commercial antigen was relatively insensitive. In the animal systems, a significant number of positive skin tests were detected with a number of experimental skin-test antigens similar to the commercial preparation. The same strain (JH) used in preparing the commercial antigen was used in the experimental series. The tissue-culture and the yolk-sac preparations seemed equally sensitive. These results were clear despite the fact that the system used was not ideal for demonstrating delayed hypersensitivity.

On the basis of these results the recommendation may be made that: 1. a pharmaceutical firm interested in the commercial production of the Frei test antigen be given the recent isolates obtained in this laboratory; 2. that after the usual screening tests for viral contamination, etc., these strains be used to prepare fresh Frei test antigens, preferably in tissue culture systems or secondarily and less favorably in yolk sacs; and 3. that these fresh antigens be used in diagnostic experiments.

Table I

Frei Test with Lygranum Type Antigens

CA-295 6 Weeks After Infection with 434

Antigen from Isolate	Yolk Sac Antigen	Tissue Culture Antigen
Normal control	----*	----
404	----	----
434	----	----
440	----	6 X 6
470	----	----
514	7 X 7	6 X 6
526	----	----
810	6 X 6	5 X 6
JH	----	6 X 7
Commercial antigen	7 X 7	
Commercial control	6 X 7	

*negative; positive reactions = measurement of papule in mm at 48 hours.

Table 2

Frei Test with Lygranum Type Antigens

CA-295 3 Months After Infection with 434

Antigen from Isolate	Yolk Sac Antigen	Tissue Culture Antigen
Normal control	----*	----
404	----	Trace
434	Small papule	Trace
440	2 X 2	3 X 3
470	2 X 2	Trace
514	4 X 4	4 X 4
526	Trace	2 X 2
810	Trace	2 X 2
JH	Trace	2 X 2
Commercial antigen	5 X 4	
Commercial control	4 X 3	

*negative; positive reactions = measurement of papule in mm at 48 hours.

Table 3

Frei Test in 6 Rabbits

6 Weeks After Infection with LGV Isolate 434

Antigen Prepared from Isolate	No. Rabbits Positive with YS Antigen	No. Rabbits Positive with TC Antigen
Normal control	0*	0
404	1	3
434	6	4
440	2	2
470	4	3
514	2	4
526	3	2
810	2	2
JH	2	2
Commercial antigen	0	
Commercial control	0	

*number of rabbits giving papular response >4 mm at 48 hours.

Table 4

Frei Test in 6 Rabbits

6 Weeks After Infection with LGV Isolate 440

	No. Rabbits Positive with	
Antigen Prepared from Isolate	YS Antigen	TC Antigen
Normal control	0*	0
404	2	0
434	0	0
440	2	1
470	0	0
514	2	2
526	2	2
810	2	2
JH		
Commercial antigen	2	----
Commercial control	2	----

*number of rabbits giving papular response >4 mm at 48 hours.

III. Serologic studies on veterans from Vietnam.

Through the cooperation of Captain Michael Benenson we received 92 serum specimens from U.S. servicemen who had spent at least 1 year in Vietnam. The original plan was to have approximately 1,000 sera for these tests, and efforts are continuing to obtain more sera in the coming year from men either in Vietnam or passing through the San Francisco area for mustering out. These 92 sera were obtained from men with and without histories of venereal disease during this period. The results of CF tests with the chlamydial group antigen are summarized in Table 5. The rate of reactors in these two groups of servicemen was not significantly different. The sera from servicemen gave 6% positive reactions at a 1:16 level and approximately 18% at 1:8 or higher. No titers obtained were on the order of magnitude normally seen in patients with clinical LGV. The rate of positives was somewhat higher than that for the normal male population in the San Francisco area, where in this age group 2-3% CF positives are expected. The slightly higher rate in the small group of patients is difficult to interpret, but if this finding stands up when more sera are tested, it will probably indicate a higher exposure to chlamydial agents. Because the CF test is group reactive, one cannot tell whether this higher rate might be due to infections with TRIC agents, which in this population would probably cause nongonococcal urethritis, or whether these patients had any significant exposure resulting in subclinical infections with LGV agents. Lacking pertinent clinical data on the patients, one cannot make these correlations. During the coming year we hope to investigate this point further by attempting to isolate chlamydiae from the genital tract of returnees reporting at Letterman Hospital for signs of urethritis. Results of this would allow us to determine prevalence of chlamydial infection and whether these agents are TRIC agents or LGV strains. Possibly either of these agents may indeed be a significant cause of nongonococcal urethritis in this population.

Table 5

LGV CF Tests in Servicemen After One Year in Vietnam

CF Titers	No. of Men with History of VD (24)		No VD (65)	
≤1:4	18 (75%)		55 (85%)	
1:8	5 (21%)	}25%	6 (9%)	}15%
1:16	1 (4%)		4 (6%)	

IV. Experimental animal infections.

Attempts to infect the conjunctiva of subhuman primates have continued and the results obtained in preliminary experiments have been confirmed. The LGV isolates are capable of producing a mild limited focal follicular conjunctivitis in monkeys. The ocular infection is self-limited but becomes systemic after dropping the agent on the conjunctiva. The ability of LGV strains to produce follicular conjunctivitis is contrary to the generally accepted belief that only TRIC agents among the chlamydiae produce follicular conjunctivitis in man or subhuman primates. The growing body of evidence suggests that the LGV strains are similar indeed to the TRIC agents, and some recent studies have shown marked antigenic cross-reactions.[3] These observations, together with those we previously made concerning the irregular pathogenicity patterns obtained with fresh LGV isolates,[4] indicate the difficulty of identifying chlamydial isolates from the human genital tract. At present some markers exist for differentiation between LGV and TRIC strains, but the lines between these two groups appear blurred and in the right individuals these strains, when properly presented, probably cause a variety of clinical diseases. This possibility will be followed up by expanding further studies to include conjunctival infections of returning soldiers.

---

[3] Wang, S. P. and Grayston, J. T.: Immunological relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test. Amer. J. Ophthal., 70: 367-374, 1970.

[4] Schachter, J. and Meyer, K. F.: Lymphogranuloma venereum. II. Characterization of some recently isolated strains. J. Bact., 99:636-638, 1969.

V. Immunologic response in experimental animal infections.

Major Vern Juchau, Medical Service Corps (in partial fulfillment of requirements for the Ph.D. degree) is collaborating in this study of immunologic response, which emphasizes the detection of specifically directed immunoglobulins after exposure to LGV antigen. It was initiated because some earlier results, obtained in Doctor E. Jawetz' laboratory, indicated a poor IgM response in monkeys infected with TRIC strains. Similar results were obtained in studies on volunteers. Because LGV is known as a disease that produces elevated gamma globulin levels in man, it seemed reasonable to attempt to assess immunoglobulin responses to LGV infection in subhuman primates and, where possible, to apply the information obtained to a study of human sera. These experiments will, of course, be reported in much greater detail in the thesis of Major Juchau, which will be submitted to the Office of the Surgeon General, U.S. Army, and published in part elsewhere. The general methods used to detect specific antibodies involve CF test with group antigen and FA tests, using either fluorocarbon purified elementary body suspensions or inclusions in infected HeLa cell monolayers as antigens. The FA and CF tests were performed on whole serum and on fractions of serum obtained by column fractionation. The specificity of the preparations was determined by the usual criteria; their purity, by radial immunodiffusion. Quantitation of immunoglobulins was determined by the latter method. The IgG and IgM preparations were quite pure; the IgA was less so, but it was also virtually nonreactive in all tests and will therefore be ignored. In the FA tests an indirect method with fluorescein-conjugated fractions of goat antihuman serum was used. Considerable variability was found in the commercial antisera prepared against IgM.

Tables 6 and 7 show a typical serologic response to LGV infection in a subhuman primate. The IgM response in fractionated serum was obtained early, and by day 7 readily detectable amounts of CF antibody were present. These antibody titers peaked early and fell off at week 5. The IgM fractions were positive before the whole serum fractions became reactive. IgG antibody levels were demonstrable earlier (14 days) by FA than by CF (35 days) and persisted long after the IgM antibody levels had become undetectable. Reinfection caused a booster reaction in both IgM and IgG levels. This test system allowed differentiation of results obtained with serum fractions and with whole serum. The above description was applicable to serum fractions in both FA and CF systems, but the CF method was more sensitive for determining IgM. The 2 tests appear to measure different antibodies. These results are all consistent with known responses to bacterial or viral infections. However, similar results were not always obtained with whole sera. In a number of instances the fractions obtained from negative whole serum were reactive. These results probably indicate some inhibition of serologic reactions by components present in the serum. Similar experiments will be performed on human sera (which have been stored whenever obtained). It is not known whether some negative or lowered CF results obtained in certain patients with LGV reflect the presence of inhibitory substances in the sera, but, if present, they would reduce the sensitivity of this test.

Table 6

Complement-fixing Antibody Levels in an African Green Monkey Following Infection with an LGV Agent*

Bled at	CF Titers in: Whole Serum	IgM	IgG
Day 0*	0**	0	0
3	0	2	0
7	0	32	0
10	0	32	0
14	0	32	0
21	0	64	0
28	16	32	0
35	16	16	4
42	16	0	8
49	16	0	16
84	8	0	
120*	0	0	0
127	16	4	
134	32	0	32
147	16	0	
171	8	0	

*_Cercopithecus_ _aethiops_ #231 infected with LGV isolate 434-L on Day 0 and Day 120.

**0 = <1:2

Table 7

Fluorescent Antibody Levels in an African Green Monkey Following Infection with an LGV Agent*

	Fluorescent Antibody Titers in:			
	Whole Serum		Serum Fractions	
Bled at	IgM	IgG	IgM	IgG
Day 0*	0**	0	0	0
3	0	0	0	0
7	0	0	0	0
10	0	0	0	0
14	0	0	32	64
21	0	8	4	16
28	0	8	0	512
35	0	256	0	256
42	0	256	0	256
49	0	128	0	ND
84	0	16	ND	ND
120*	0	0	0	0
127	0	0	0	0
134	0	16	16	256
147	0	128	0	256
171	0	32	0	64

*Cercopithecus aethiops #231 infected with LGV isolate 434-L on Day 0 and Day 120.

**0 = <1:2

VI. Serotyping of lymphogranuloma venereum isolates.

Attempts to develop a method of serotyping LGV isolates by neutralization tests are continuing. Briefly, the technique used involves assay of infectivity, in plaque-forming units on an L-929 cell monolayer.[5] The plaque reduction test measures neutralizing antibody.[6] Because human and other mammalian sera do not contain demonstrable neutralizing antibodies after infection, the source of hyperimmune serum has been the rooster. Preliminary results were included in the 1970 Annual Report, and an expanded series is presented in Table 8. This system has been found suitable for testing with the 9 LGV isolates, 2 "fast" TRIC strains, and the mouse pneumonitis strain (Nigg),[5] all members of the species Chlamydia trachomatis. Neutralizing antiserum has been developed against 6 LGV isolates, 1 "fast" TRIC strain, and 1 LGV-like isolate (678) obtained from the blood of a woman with a febrile disorder. None of these sera reduced infectivity of the mouse pneumonitis strain indicating marked antigenic difference. Cross reactivity among the other isolates was marked and to date it has not been possible to divide this group into serotypes on the basis of these results, which are still to be considered preliminary. Further rooster immunization experiments are in progress in an attempt to obtain higher titered antiserum to those isolates which yielded no neutralizing antibodies or low titers initially.

The LGV isolates obtained in this study have been circulated to other interested research workers, and a recent publication indicates that demonstrable serotypes do exist within this group.[3] The use of a microimmunofluorescent technique (MIF) with acute phase mouse antiserum, has revealed small but reproducible differences. Three serotypes are indicated, and isolates 404, 434, and 440 represent each type. This MIF test has also shown very marked cross reaction among all the LGV isolates tested and the serotypes are based on reproducible differences in reciprocal crosses. Results in this test appear to parallel those obtained in mouse toxicity prevention tests.

When neutralizing antisera to all the LGV isolates are obtained it will be of considerable interest to see whether plaque reduction results are parallel to the MIF. If so, it would indicate that the toxicity prevention antigen-antibody system is similar to the infectivity neutralizing antigen-antibody system.

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[5] Banks, J., Eddie, B., Schachter, J., and Meyer, K. F.: Plaque formation by Chlamydia in L cells. Infect. Immun., 1:259-262, 1970.

[6] Banks, J., Eddie, B., Sung, M., Sugg, N., Schachter, J., and Meyer, K. F.: Plaque reduction technique for demonstrating neutralizing antibodies for Chlamydia. Infect. Immun., 2:443-447, 1970.

[3] Wang, S. P. and Grayston, J. T.: Immunological relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test. Amer. J. Ophthal., 70: 367-374, 1970.

Table 8

Plaque Reducing Titers of Antiserum Against LGV and Similar Isolates

Infecting Isolate	Serum Against: 404	440	434	470	678	526	514	LB1
404	76*	160	15		16			
440	21	447	16	19				40
810		148	33	20	26			25
434	24	54	145		22			
470	25	74	28	17	14			
JH		33	31	30	13			63
678		40	40	36	46			40
526		160	68	18	12	17		105
514		22	115			20	32	
LB1					22			17
T'ang					16			10

*dilution of serum giving 50% reduction in plaque count.

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4. Schachter, J. and Meyer, K. F.: Lymphogranuloma venereum. II. Characterization of some recently isolated strains. J. Bact., 99: 636-638, 1969.

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6. Banks, J., Eddie, B., Sung, M., Sugg, N., Schachter, J., and Meyer, K. F.: Plaque reduction technique for demonstrating neutralizing antibodies for Chlamydia. Infect. Immun., 2:443-447, 1970.

APPENDIX I

Preparation of LGV - Skin Test Antigens

A. Yolk sac material

Treat normal yolk sac and pools of 8 isolates (404, 434, 440, 470, 514, 526, 810 and JH) in the same manner:

1. Harvest yolk sacs, check smears and sterility, discard contaminated or questionable smear YS.
2. Prepare 10% suspension in saline (Waring Blender).
3. Centrifuge in cold, 1 hour low speed (approximately 500 rpm); save supernate; discard sediment.
4. Divide supernate into 2 equal portions for further processing.
5. Centrifuge both supernates in Servall, in cold, at 17,000g for 20 minutes; discard supernates.
6. From this step on the 2 sedimented elementary body preparations will be handled separately; label one as "isolate number: Lygranum", and label the other as "isolate number: acid extract".
7. Lygranum type: resuspend the sediment in 0.1% sterile formalin saline to a final volume equal to 1% YS (10X starting volume); store in refrigerator for 48 hours; add phenol (0.25%) final and dispense into vaccine bottles; sterility test and store at 4° C.
8. Acid extract: suspend sediment to original volume with sterile saline; add HCl to final concentration 0.02N and place in 37° water bath for 30 minutes (if precipitate forms, remove by low speed centrifugation and keep); neutralize with NaOH (spin off precipitate and keep); dispense into vaccine bottles; sterility test and store at 4° C.

B. Tissue Culture material

Treat normal HeLa cells and agent pools in the same manner; use 5 day infected monolayers in 32 oz. bottles:

1. Check smears and sterility; titrate in eggs.
2. Homogenize harvest material.

3. Low speed centrifugation to remove cell debris.

4. Divide into 2 equal portions (Lygranum and acid extract).

5. High speed centrifugation to sediment elementary bodies.

6. Lygranum type: resuspend to original volume in 0.1% formalin saline and treat as egg material.

7. Acid extract: resuspend to 1/10 original volume and treat as egg material.

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13. ABSTRACT

Only 6 new lymphogranuloma venereum (LGV) cases were seen this year. The reduced number may reflect an actual decrease or may indicate fewer referrals to the San Francisco hospitals.

Experimental Frei test antigens were tested in LGV-infected rabbits and monkeys. The commercial Lygranum antigen, which has been a relatively poor diagnostic tool in humans, was included. It was also a poor skin test antigen in the experimental system. This system was far from ideal, but positive tests of delayed hypersensitivity were obtained with a number of experimental antigens prepared from recently isolated LGV strains. It is suggested that development of an improved antigen, possibly including recent isolates, be undertaken.

Complement fixation (CF) tests, performed on the sera of 92 servicemen spending at least a year in Vietnam, yielded a 6% rate of positives. This rate is approximately twice the rate in a young male civilian population but the size of the sample is too small to draw any conclusions. Studies in progress, of experimental LGV infections and methods for serotyping LGV isolates, are described.

Serologic response in experimental LGV infection has been studied. The time sequence of specific immunoglobulins has been studied. Anomalous results obtained with whole serum and fractions indicate the possible presence of inhibitors of CF in whole serum. This may be significant in the use of CF as a diagnostic test.

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CELLULAR AND HUMORAL IMMUNITY IN PARASITIC INFECTIONS (U)

ANNUAL PROGRESS REPORT NO. 2

by

Noel R. Rose, M.D.,Ph.D.

(For the period 1 June 1970 to 31 May 1971)

Supported by

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SUMMARY

The purpose of the research described in this report is to determine the interrelationships of the several manifestations of the immune response in parasitic infections. It is apparent from the results described that there is a distinct delayed-hypersensitive response to toxoplasma infection in both guinea pigs and man as measured by macrophage inhibition, lymphocyte inhibition and cytotoxicity.

While the extent of delayed-hypersensitivity as measured by macrophage inhibition was seen to increase with time no such relationship was seen with lymphocyte transformation. In addition, whereas the extent of macrophage inhibition increased with increasing antibody titers there was an inverse relationship between antibody titers and lymphocyte transformation.

Toxoplasma antigen has been shown to be stimulatory to macrophage migration in some normal individuals and to be mitogenic to the lymphocytes of some normal individuals. In addition, toxoplasma infection results in a lymph node morphology, level of lymphocyte transformation, and a monopolization of the humoral system indicative of a strong immunogen.

FOREWORD

The results of the research described in this report are based upon data from both human and animal subjects. In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animals and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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I. Specific Aims:

Specific acquired resistance to animal parasites has been variously associated with different types of humoral antibodies, phagocytic cells of several classes, or immediate or delayed hypersensitivity reactions. All of these components of the immune response may be raised during parasitic invasion. The aim of the present proposal is to study the inter-relationships of the several manifestations of the immune response in an effort to: (1) gain more insight into the basis of clinical immunity in parasitic infections; (2) develop practical techniques for assessing the immune status of the host; (3) project possible methods for enhancing specific immunity; and (4) seek improved methods for immunological diagnosis of parasitic infection. The primary hypothesis of the first phases of the work is that cell-mediated immunity plays a major role in the specific acquired resistance to tissue parasites.

II. Background:

While circulating antibodies can easily be demonstrated in most parasitic infections and frequently reach high levels, there is generally a poor correlation between antibody titer and specific acquired resistance. Moreover, the many serological techniques that can be applied often demonstrate different patterns of antibody response. If circulating antibody does not play the key role in immunity to tissue parasites, it is possible that cell-mediated immune responses are paramount. For example, present or past leishmanial infection is associated with the development of delayed hypersensitivity as measured by the leishmanin test. In trypanosomal infection, also, there is suggestive evidence that although circulating antibodies may be important for the blood stages of the infection, it does not affect the tissue phase of the organism. It would seem reasonable, therefore, to investigate all parameters of the immune response in a carefully selected model of intracellular parasitic infection in order to clarify the role of various types of humoral antibody, of phagocytic mechanisms, or of delayed hypersensitivity in producing effective immunity.

Experimental toxoplasmosis was selected as a suitable model for the initial studies. Toxoplasma gondii is an ubiquitous parasite of homeotherms including man. In experimental infections, there is rapid proliferation of the parasitic cells in the reticuloendothelial system corresponding to an acute stage of infection. It is followed by a cystic phase of intracellular parasitism. In human adults, the acute disease is rare, but antibodies are common, suggesting an effective immune response. The current status of diagnostic and clinical knowledge of this disease has been summarized by Feldman (1). The present state of our knowledge of Toxoplasma gondii with respect to its life cycle, morphology, mechanism of transmission, and association with various disease conditions has been reviewed by Jacobs (2). In addition, the complete life cycle of toxoplasma has recently been elucidated by the discovery of the missing sexual stage in the gut mucosa of cats (3).

It is relatively easy to infect laboratory animals with this organism and standardized infections can be obtained with well-characterized strains of the parasite. In addition to their ready cultivation in experimental animals, toxoplasma can be grown on cultured human cells. For example, HEP_2 (4) and HeLa (5) have been used.

III. Approach to the Problem:

The major portion of this project is designed to investigate the extent to which the mechanisms of cellular hypersensitivity are involved in immune response to toxoplasma. Current immunological investigation has provided three suitable reproducible, and quantitative methods for assaying cellular hypersensitivity. These are macrophage inhibition as elaborated by David (6); the cytotoxic effect of lymphoid cells as applied by Govaerts (7), Rosenau and Moon, (8), and Rose et al., (9); and lymphocyte transformation as employed by Caron et al., (10) and Girard, Rose et al., (11). All three of these methods provide complimentary information about the cell-mediated aspects of the immune response.

Because of the ease with which delayed hypersensitivity can be demonstrated, the similarity to the human d-h response, the short time needed before detection of a d-h response, and its use as the animal best suited for macrophage inhibition studies, we have chosen the guinea pig as our experimental animal. Once a technique was applied to this system with success, we then have applied it to cases of human toxoplasma infection or sensitivity. While the number of patients with active or past toxoplasma infection is limited, the data from only a few such individuals is sufficient to indicate whether the data from our experimental animal system is a valid model of human infection and immunity.

In all instances, both experimental animal and human patients, the indirect fluorescent antibody test was used to determine the antibody titers of the individuals. In addition, in cases of human infection the titers of the specific immunoglobulins (IgG, IgM, IgA) were determined. Data from this aspect of the research when combined with the investigations of cellular immunity will hopefully elucidate the relationship between the humoral and cellular response in toxoplasma infection.

IV. Results:

A. Cell-mediated Immunity to Toxoplasma gondii as Measured by Macrophage Inhibi

1. Toxoplasma antigen sensitized guinea pigs.

Eight guinea pigs sensitized with 100 µg of toxoplasma antigen combined with complete Freund's adjuvant were skin tested with 25 µg of toxoplasma antigen from 19-37 days following sensitization. All animals showed distinct, positive skin tests with 1 to 1.5 cm erythema and induration at 24 hours (Table 1). The reactions were in each instance still distinct at 48 hours with no decrease in the reaction as compared with 24 hours. There was no distinct correlation between degree of positivity (1 - 1.5 cm) and the time elapsed between sensitization and skin testing.

Three of the guinea pigs had skin tests that were 1.5 cm in diameter at 24 hours (Nos. 67, 66, 69). These same guinea pigs showed 47, 50, and 27% inhibition of migration and had serum titers of 256, 64, and 256, respectively in the indirect fluorescent antibody test. The remaining five guinea pigs had skin tests 1 cm in diameter, their macrophage inhibition ranged from 34 to 50% and their serum titers from 256 to 4096. From this data there is no apparent correlation between the intensity of the skin test reaction and the extent of macrophage inhibition or serum titer in toxoplasma sensitized guinea pigs.

These toxoplasma sensitized guinea pigs were sacrificed between 27 and 93 days following sensitization and the extent of macrophage inhibition determined (Table 1). The extent of migration inhibition of the peritoneal macrophages in the presence of 100 µg/ml toxoplasma antigen ranged from 27 to 50%. As can be seen from Table 1, between 27 and 93 days after sensitization there is no indication of a trend toward greater inhibition as a function of time. In fact, with the exception of animal number 69, these eight toxoplasma sensitized animals showed little variability in their macrophage inhibition (34 to 50%).

From these data it would appear that the cellular response is well established by 4 weeks following sensitization and does not significantly change through 12 weeks.

While the percent of inhibition was in each case considerably above 20% which is generally considered the minimum to be considered valid inhibition, it is apparent from Table 1 that the t-test indicates the results in two instances, animals 66 and 69, show rather high probability of similarity (<.05 and <.1 respectively) between migration in control chambers and chambers containing toxoplasma antigen. In the

Table 1.

Toxoplasma antigen sensitized guinea pigs

Guinea Pig No.	Skin test results		Duration of sensitization	Macrophage inhibition results		Serum titer (IFA)*
	days post sensitization	diameter		Percent inhibition	t-test	
46	23	1 cm	27 days	34	TXC-$\leq$.001**	256
67	19	1.5 cm	28 days	47	TXC-$\leq$.01	256
43	23	1 cm	29 days	43	TXC-$\leq$.001	256
40	23	1 cm	32 days	46	TXC-$\leq$.001	1024
66	19	1.5 cm	32 days	50	TXC-$\leq$.05	64
69	19	1.5 cm	32 days	27	TXC-$\leq$0.1	256
72	37	1 cm	60 days	50	TXC-$\leq$.001	4096
50	63	1 cm	93 days	44	TXC-$\leq$.001	4096

* IFA - indirect immunofluorescent antibody. Antibody was determined on the day of sacrifice.

** T - Chamber with toxoplasma antigen.
C - Control chamber with no antigen.

case of the other six guinea pigs in this group the probability of similarity (<.001) is extremely small. In each instance there was no inhibition of migration in the presence of the unrelated antigen Entamoeba, histolytica, t-test analysis indicating significance at levels in excess of 5%.

2. Toxoplasma infected guinea pigs

Eight guinea pigs were infected with 10 toxoplasma organisms intramuscularly for the purpose of producing a non-lethal, chronic toxoplasma infection. No skin tests were performed on these animals to avoid the possibility of altering their immune status. These animals were sacrificed between 11 and 200 days following infection and inhibition of macrophage migration in the presence of toxoplasma antigen and an unrelated antigen (Entamoeba, histolytica) measured. The results (Table 2) indicate that with the exception of one animal, number 74, inhibition of macrophage migration occurred in the presence of toxoplasma antigen. Guinea pig number 70 which was infected for only 11 days before evaluation of macrophage inhibition showed 26% inhibition while guinea pig number 106 which was infected for 200 days showed 79% inhibition. While the correlation between duration of infection and extent of migration inhibition is not absolute, it can be seen from Table 2 that there is a distinct trend toward greater inhibition of migration with increased duration of infection. The reason for the lack of inhibition in the case of animal number 74 was not apparent as the presence of an antibody titer of 4096 confirmed that the animal had been infected.

As can be seen from Table 2, t-test analysis of these data indicate significance at the 1% level or less in six of the eight animals. The extent of inhibition in the case of guinea pig number 70 approached significance at the 5% level which is consistent with the minimal level of inhibition (26%). T-test analysis in the case of guinea pig number 74 which showed no inhibition of migration indicated no significant differences between control and experimental groups. In each instance there was no significant inhibition of migration in the presence of the unrelated antigen, Entamoeba histolytica.

3. Normal guinea pigs

Six unsensitized and uninfected guinea pigs were used in macrophage inhibition tests with toxoplasma antigen. The results in Table 3 show that in no instance were the normal guinea pigs macrophages significantly inhibited in the presence of toxoplasma antigen. Of the six animals only numbers 11 and 55 showed inhibition (6 and 12% respectively) which is

Table 2.

Toxoplasma infected guinea pigs

Guinea Pig No.	Duration of infection	Percent inhibition		Serum titer (IFA)
70	11	26	EXC >.05 TXC ≤.05	64
68	12	38	EXC >.05 TXC ≤.001	1025
59	18	43	EXC >.05 TXC ≤.01	1024
35	22	35	EXC >.05 TXC ≤.01	256
74	53	0	EXC >.05	4096
107	56	49	EXC >.05 TXC ≤.001	256
106	200	79	EXC >.05 TXC ≤.001	4096
112	200	57	EXC >.05 TXC ≤.001	4096

Table 3.

Normal guinea pigs

Guinea Pig No.	Serum titer (IFA)	Percent inhibition	t-test
76	0	35% stimulation	
11	0	6%	TXC >.05
12	0	63% stimulation	
23	0	33% stimulation	
55	0	12%	TXC >.05
85	0	7% stimulation	

considerably below the 20% considered necessary. The remaining 4 showed stimulation of migration in the presence of toxoplasma antigen as compared to the control chambers. In one instance, number 12, the stimulation in the presence of toxoplasma antigen was 63% as compared to 35%, 33% and 7% in the other animals that showed stimulation. In two instances of slight inhibition, 2 and 12%, the t-test data indicate significance at levels considerably in excess of 5%.

4. Toxoplasma infected humans.

Two individuals with significantly elevated IFA titers to toxoplasma (256 and 1024) have been studied using macrophage inhibition.

Patient	IFA titer	% inhibition
A.G.	256	13
Z.M.	1024	30

Patient A.G. with a titer of 256 was asymptomatic and, as can be seen from the table, has only 13% inhibition which is considerably below the 20% level which is considered the minimum significant inhibition. Patient Z.M., however, is currently suffering from chorioretinitis, fatigue, and chest congestion. Several years ago this patient experienced a period of fever, muscle weakness, and loss of consciousness. The IFA titer of this patient is rather high and there was significant macrophage inhibition (30%).

5. Normal humans

Two healthy normal individuals have been examined for macrophage inhibition in the presence of toxoplasma antigen.

Patient	IFA titer	% inhibition
R.H.	0	50% stimulation
M.L.	0	0

In both instances, there was no inhibition of migration although there was a 50% stimulation of migration in the presence of toxoplasma antigen in the case of patient R.H. Stimulation of normal individuals was also a feature of the experiments carried out in guinea pigs (IV., A., 3).

B. Cell-mediated Immunity to Toxoplasma gondii as Measured by Lymphocyte Transformation

1. Toxoplasma infected guinea pigs

Eleven guinea pigs were infected with 10 toxoplasma organisms given intramuscularly in the same manner as those used for the macrophage inhibition study. These animals were sacrificed between 12 and 242 days following infection and their blastogenic response in culture to toxoplasma antigen determined by the uptake of tritiated thymidine. Three of these animals, 63, 106, and 112, were also used for macrophage inhibition (see Table 2). The results (Table 4) indicate an increase in lymphocyte transformation over similar cultures in the absence of toxoplasma antigen of from 1.7 to 53 times. From this table it can be seen that the amount of transformation is minimal (1.7 - 3 times controls) during the first 3 weeks. Beyond 3 weeks following infection the amount of transformation ranges from 6 to 53 times control values though there appears not to be a trend toward increasing transformation with duration of infection.

However, if the amount of transformation is compared to the antibody titers of the animals (Fig. 1), with one exception (animal number 128 with 53 times control value for LT), one sees a distinct inverse relationship between the amount of lymphocyte transformation and the antibody titer in the range beyond 5 times control lymphocyte transformation values. The significance of this range above 5 times control values will be seen in the following section on the lymphocyte transformation response to toxoplasma antigen in normal animals.

2. Normal guinea pigs

The peripheral lymphocytes of ten uninfected guinea pigs were examined for their response to toxoplasma antigen in culture. The results (Table 5) indicate that the toxoplasma antigen will cause low levels (up to 4X control values) of transformation in normal guinea pigs. Of the ten normal animals, five (55, 164, 165, 178, 207) showed increases of lymphocyte transformation above control values while the remaining five showed no stimulation in the presence of toxoplasma antigen.

3. Toxoplasma infected humans

Two individuals with significantly elevated IFA titers to toxoplasma (256 and 1024) were examined for the ability of their lymphocytes to transform in the presence of toxoplasma antigen.

Table 4.

Lymphocyte transformation in toxoplasma infected guinea pigs

Guinea Pig No.	Lymphocyte transformation (X control)	Days of infection	Antibody titer*
63	1.5	12	1024
190	3	12	16
96	3	22	0
32	20	26	1024
99	28	29	1024
113	6	101	8192
123	53	105	4096
108	22	116	256
106	10	200	8192
112	16	200	4096
129	24	242	256

* Indirect fluorescent antibody test

Relationship between lymphocyte transformation and antibody titer

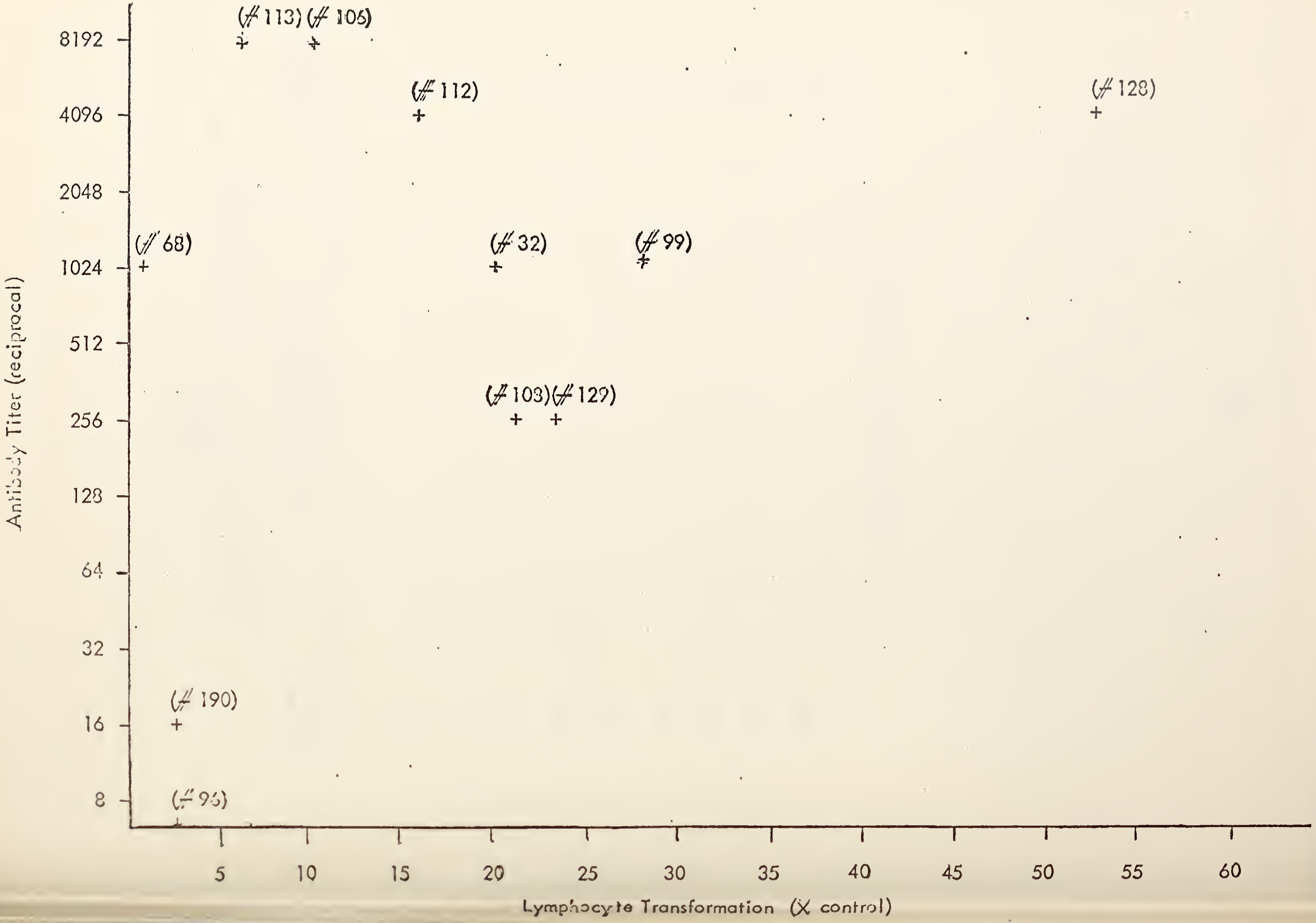

Table 5.

Lymphocyte transformation in normal guinea pigs

Guinea Pig No.	Lymphocyte transformation (X control)	Antibody titer*
X	0	0
139	0	0
35	0	0
55	4	0
130	0	0
164	1.7	0
135	2	0
178	3	0
183	0	0
207	3	0

* Indirect fluorescent antibody titer

Patient	IFA titer	% inhibition
A.G.	256	70X
Z.M.	1024	140X

Patient A.G. was asymptomatic while patient Z.M. (discussed in section IV., A., 4) had evidence of active infection. From the above table it can be seen that in these two instances there is a direct relationship between IFA titer, activity of disease, and amount of lymphocyte transformation.

4. Normal humans

Two healthy normal individuals (the same as used for macrophage inhibition in section IV., A., 5) were evaluated for the ability of their lymphocytes to transform in the presence of toxoplasma antigen.

Patient	IFA titer	Lymphocyte transformation X control values
R.H.	0	20
M.L.	0	2

One of these individuals, M.L., showed minimal stimulation of 2 times control values while the other showed stimulation of 20 times the control value. This is similar to the apparent mitogenic effect of toxoplasma antigen seen in the studies on normal guinea pigs (section IV., B., 2).

C. Cell-mediated Immunity to Toxoplasma gondii as Measured by the Cytotoxicity of Immune Cell Populations.

We initially attempted to measure cytotoxicity to Toxoplasma gondii by culturing the toxoplasma in the presence of Cr^{51} and subsequently measuring the release by the toxoplasmas of the Cr^{51} in the presence of immune cell populations. Problems in getting the toxoplasmas to take up Cr^{51} and retain it have not been completely solved so the initial stages of our investigation of cytotoxicity have been carried out using the trypan blue dye exclusion test as an indication of toxoplasma viability in the presence of immune and non-immune cell populations (spleen cells, mesenteric lymph node cells, peritoneal exudate cells, peripheral blood lymphocytes).

In the presence of normal cell populations we have found that the number of toxoplasmas present in the cultures decreases considerably in the presence of the peritoneal exudate cell population and less so in the presence of the other cell populations over a 6 hour period. In the presence of cell populations from toxoplasma infected animals the number of intact toxoplasmas decreases sharply between 1 and 3 hours of incubation in the presence of peripheral blood leucocytes and peritoneal exudate cells while the number of intact toxoplasmas changed little in the presence of spleen cells and lymph node cells. Also in the presence of cell populations from infected animals, of the toxoplasmas that are intact the number that are dead as determined by their inability to exclude the trypan blue dye doesn't significantly change in the presence of mesenteric lymph node cells; however, the number of dead toxoplasmas does increase with time in the presence of peripheral blood leucocytes and peritoneal exudate cells.

D. The Effect of Toxoplasma Infection on a Subsequent Immune Response.

Sprague-Dawley rats were divided into four groups: Group 1 was infected intraperitoneally with 1000 toxoplasma organisms, Group 2 was treated in the same way and ten days later was given 1 ml of a 2% sheep red blood cell (SRBC) suspension i.v., Group 3 was only given the sheep cells, and Group 4 were normal, untreated animals. All four groups were sacrificed four days following the SRBC injections and the indirect fluorescent antibody titer to toxoplasma determined, the hemolytic antibody titer to SRBC determined, and the rat spleen cells assayed for plaque forming cells, Within the framework of this single antigen the results indicate that infection with toxoplasma almost totally suppress the rats ability to respond to the SRBC challenge with either hemolytic antibody or a plaque forming cell population. This particular phase of our investigation of immunity to parasitic infections was prompted by the architecture and cell population of lymph node cells from toxoplasma infected guinea pigs. Such nodes showed a decrease in germinal centers and were monopolized by large aggregations of lymphocytes in cord-like patterns.

V. Discussion of Results:

A. Cell-mediated Immunity to T. gondii as Measured by Macrophage Inhibition

The results of our study of macrophage inhibition as an indicator of delayed hypersensitivity in toxoplasma sensitized and toxoplasma infected guinea pigs indicate that while no correlation between extent of macrophage inhibition and duration of sensitization can be made, there is a distinct relationship between extent of inhibition and duration of infection. It would appear that our infecting dose of 10 toxoplasma organisms intramuscularly was small enough to allow the animal to deal with the infection and in most instances keep the infection under control. Subsequent to such a "balance" being established, it would appear that the constant presence of the organisms resulted in an increasing response as evidenced by both the rising titer and the increasing delayed hypersensitivity as measured by macrophage inhibition.

From our results it would appear that an IFA titer of 4096 is near the maximum (the highest we have seen in such infected animals is 8192) while the macrophage inhibition seen in animals infected for 200 days (57 and 79%) likely has not approached a maximum. Our toxoplasma antigen sensitized animals did not show this progression of delayed hypersensitivity, presumably due to an initial period during which the immune system was stimulated by the deposited antigen with no increasing stimulation due to proliferation and no continual stimulation due to chronic infection as in the case of the infected animals.

While a chronic toxoplasma infection without overt symptoms appears to represent a balance the toxoplasma infection and the immune response of the individual, it is doubtful that a state of balance really exists. Rather, the host is gradually compromised by the effects of the chronic infection particularly by damage to the tissues in which the toxoplasma is residing and possibly by an impaired ability to respond to super-imposed antigenic challenge, a point that will be discussed later. This subliminal attack of the toxoplasma is evidenced by the lesions in the brain and/or heart of chronically infected humans (12). Such an individual has many pseudocysts in the brain and a lesser number of areas showing monocytic infiltration accompanied by focal necrosis. Such areas of infiltration and necrosis likely represent locations where pseudocysts have ruptured resulting in the lesions due to a delayed hypersensitive response to the free organisms. Apparently the cysts are not antigenic as cysts are rarely found in areas of infiltration (13).

A noteworthy effect observed in the course of these experiments was the stimulatory effect of toxplasma antigen on migration in the case of the normal guinea pigs and human subjects. Four of the six normal guinea pigs and one of the two normal patients showed stimulation of migration. Using a leucocyte migration system with B. abortus as the antigen, Søborg (14) noted stimulation at low antigen concentrations with cells of moderate to low sensitivity. As we have found no evidence of previous contact with toxoplasma in both our normal guinea pigs and humans, it is doubtful that we are seeing the same phenomenon.

Concerning the relationship between the IFA titers and the extent of macrophage inhibition in the infected guinea pigs, it is apparent that the IFA titers rose faster in the infected animals than the sensitized ones; however, by 60 days post sensitization or infection, it would appear that a near maximum titer of 4096 is reached. The apparent lack of correlation between the extent of migration inhibition and the IFA titer in the sensitized or infected animals combined with the minimal role shown for circulating antibody in immunity to toxoplasma by Frenkel (15), re-emphasizes the fact that antibody titers cannot be taken as an indication of the status of an individual's immunity to toxoplasma infection. Hopefully, as we investigate the delayed hypersensitive response by macrophage inhibition in more instances of human infection, this statement can be applied to human infection with equal confidence.

B. Cell-mediated Immunity to Toxoplasma gondii as Measured by Lymphocyte Transformation.

Contrary to the relationship which appears to exist between the antibody titer and extent of macrophage inhibition in infected guinea pigs, the relationship between antibody titer and extent of lymphocyte transformation in the presence of toxoplasma antigen is an inverse one as can be seen from Fig. 1. Also, unlike macrophage inhibition the extent of lymphocyte transformation does not increase with duration of infection, a fact which can be inferred from the inverse relationship with the titer. The possibility exists that the two events are competitively linked in some fashion; however, additional investigations would be necessary to clarify this point.

In the same fashion that toxoplasma antigen was found to be stimulatory to macrophage migration in normal guinea pigs and humans it was found to be mitogenic in some normal guinea pigs and humans. Five of the ten normal guinea pigs examined and both normal human subjects showed some degree of lymphocyte transformation in the absence of any evidence to indicate a sensitivity to toxoplasma. In all instances the non-specific stimulation was considerably less than that seen in infected animals. This non-specific stimulation combined with the relatively high levels of stimulation seen in infected guinea pigs and man correlate well with the histological picture of the lymph nodes from infected guinea pigs. Such lymph nodes are often packed with cords of lymphocytes to the point where the normal architecture of the node is destroyed. These two observations may well be related to the observed immunosuppression coincident with toxoplasma infection to be discussed in section V., D.

C. Cell-mediated Immunity to Toxoplasma gondii as Measured by the Cytotoxicity of Immune Cell Populations.

It would appear from our initial studies that peripheral blood lymphocytes and peritoneal exudate cells (approximately 15% lymphocytes and 20% macrophages) from infected guinea pigs are considerably more cytotoxic for toxoplasmas than spleen cells or mesenteric lymph node cells. However, we have noted that the numbers of toxoplasmas in culture with normal peritoneal exudate cells decreases sharply. This combined with observations of toxoplasmas within peritoneal macrophages indicates that the toxoplasmas are being non-specifically phagocytized or else themselves are preferentially entering these cells.

The cytotoxic effect of the peripheral blood lymphocytes does appear to be specific and confined to infected animals. The cytotoxic effect of these cells is well underway at 4 hours of incubation and is extensive at 6 hours. Further purification of cell populations combined with other assays for cytotoxicity will provide added data and will permit the investigation of the relationship of other parameters of toxoplasma infection to be correlated with cytotoxicity including the other assays for cell-mediated immunity.

D. The Effect of Toxoplasma Infection on a Subsequent Immune Response.

Our results indicate that active toxoplasma infection in Sprague-Dawley rats apparently so monopolizes the animals immune system that they respond poorly, if at all, to the antigenic stimulus of sheep red blood cells with the production of anti-SRBC antibodies. At the same time the rats have IFA titers to toxoplasma of from 128 to 1028 ten days following infection.

The effect of an active toxoplasma infection on a subsequent delayed hypersensitive response has not yet been determined. Rats that have been so infected have been kept for six months with no apparent ill effects.

These results indicate that toxoplasma is a strong immunogen resulting in a fast and distinct humoral response and from our work with guinea pigs a distinct delayed hypersensitive response also. Whether such a strong response is beneficial, especially the humoral response, which is of doubtful value to the infected individual is questionable.

VI. Conclusions:

1. Toxoplasma infection results in a delayed hypersensitivity to toxoplasma as measured by macrophage inhibition, lymphocyte transformation, and cytotoxicity.

2. There is a positive correlation between duration of toxoplasma infection and the extent of delayed hypersensitivity as measured by macrophage inhibition.

3. There is an inverse relationship between IFA antibody titer for toxoplasma and the extent of lymphocyte transformation.

4. In the case of macrophage inhibition toxoplasma antigen was found to be stimulatory in some normal guinea pigs and humans. Toxoplasma antigen was found to be mitogenic for some normal guinea pigs and human lymphocytes.

5. Of the four cell populations investigated to date peripheral blood lymphocytes appear to be the most cytotoxic for toxoplasmas. Macrophages from normal and infected animals engulf or are penetrated by toxoplasmas to a considerable degree in culture.

6. Active toxoplasma infections in rats results in a near complete suppression of the humoral antibody response to subsequent sheep red blood cell stimulation. This apparent monopolization of the humoral response is of doubtful value to the infected individual.

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13. ABSTRACT

A distinct delayed hypersensitive response to infection with a representative intracellular parasite, Toxoplasma gondii, in guinea pigs has been demonstrated using three methods of assessing the cellular immune response: inhibition of macrophage migration, lymphocyte transformation, and cytotoxicity. These data are compared and discussed in terms of the humoral response as determined by the indirect fluorescent antibody technique. A small number of human infections have been studied and are also discussed. The effect of active toxoplasma infection on the normal immune response to sheep red blood cell challenge in rats has also been studied.

The purpose of the research described in this report is to determine the inter-relationships of the several manifestations of the immune response in parasitic infections. The primary hypothesis of this first phase of the research is that cell-mediated immunity plays a major role in the specific acquired resistance to tissue parasites.

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DEVELOPMENT OF THE IMMUNE RESPONSE (U)

ANNUAL PROGRESS REPORT

by

Arthur M. Silverstein, Ph.D. and Robert A. Prendergast, M.D.

July 1971

(For the period 1 June 1970 to 31 May 1971)

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BODY OF REPORT

Problems:

These investigations are broadly aimed at identifying the factors involved in the development of immunologic competence in the mammalian fetus, in order to improve our understanding of the basic mechanisms underlying the response of the host to immunization. Among the specific objectives of these studies are: a) to identify the mechanisms of cellular differentiation whereby the hitherto immature fetus achieves competence to respond to antigenic stimulus; b) to evaluate the relationship between immunogenesis and lymphogenesis in the developing animal; c) to study those factors (especially specific antigenic stimulus) which may control both immunologic and lymphoid development; and d) to evaluate the biological implications of the achievement of immunologic competence by the fetus, in such areas as the rejection of tissue transplants, the induction of immunologic tolerance, and the alteration of host-parasite relationships which may modify the pathogenesis of certain congenital infectious diseases.

Background and Approaches:

The development of surgical techniques permitting repeated access to the fetus in utero without interruption of pregnancy has opened the door to a broad study of the ontogenesis of the immune response. The fetus is now available for immunologic manipulations of the type employed in standard experimental laboratory animals, including primary and booster immunizations and subsequent bleeding, tissue transplantation and subsequent biopsy, the implantation of catheters, and such major procedures as thymectomy and gut removal or resection. Application of these approaches to the fetus in utero (which enjoys the almost unique status of being an immunologically virgin animal and, in the sheep, an agammaglobulinemic host) permits certain types of experiments which could not otherwise be accomplished.

Previous reports have described our general findings that immunologic competence develops in the fetus in a stepwise fashion as gestation proceeds, first to one antigen and then another, etc. In addition, although there is visible no prior morphologic sign of the attainment of immunologic competence, active response to an antigenic stimulus is accompanied by the induction of a precocious maturation of the organized lymphoid tissues in the immunized host. Preliminary studies on the thymectomy of the fetal lamb suggested that this organ is less important for the maturation of immunologic competence in this species than in other species studied. Assessment of the role of the Peyer's patches as possibly exercising a control function in the mammal analogous to that of the avian Bursa of Fabricius showed that extirpation of these structures along with the entire intestinal tract fails to interfere with the orderly development of immunologic competence in the fetal lamb. The role of the immunologic status of the host in the pathogenesis of certain congenital infectious diseases was studied, employing the bluetongue virus of sheep as a model. Early studies suggested that the nature of the disease does in fact vary significantly with the stage of development of the fetal host. Employing the approaches described above, we have extended and expanded upon these studies during the past year. In addition, we have developed and explored several new experimental models designed to study the factors which control immunocyte differentiation and the regulation of those genes governing immunoglobulin formation.

Results and Discussion:

A. Ontogeny of the production of different immunoglobulin classes and subclasses. As outlined in earlier reports, the fetal lamb *in utero* develops immunologic competence to form specific antibodies against a variety of antigens at different stages of gestation. The capacity to respond to certain antigens arises very early in gestation, to others only later during development, and to yet others only sometime after birth. In all instances thus far studied, it was found that once the fetus commenced its response to any given antigen, it would produce γM and γG_1 antibodies almost simultaneously at the outset, with the formation of γG_2 antibodies of the same specificity lagging the others by perhaps a week or more. The γG_2 lag does not appear to be peculiar to the fetus, since it has been observed also in the adult. The formation of γA antibodies has not generally been observed following standard immunization procedures in the fetal lamb, a finding in accord with the results reported in other species. To test whether this failure of production of γA antibodies reflects a true delay in the maturation of the so-called secretory immunologic system, or alternatively the inability of antigen to stimulate appropriate cells in appropriate locations, we have attempted to stimulate secretory γA antibody at different stages of gestation by feeding the antigen into the gastrointestinal tract of the fetus through indwelling catheters implanted into the fetal stomach and exiting through the uterus and maternal skin. Preliminary evidence obtained with the technique of radioactive amino acid incorporation into short-term tissue cultures of the antibody-forming intestine suggests that in fact γA antibody may be formed by appropriate direction of the antigenic stimulus. Future studies to confirm this are being planned with fluorescent-labeled anti-immunoglobulin reagents, while immunization at different stages of gestation with different antigens will establish whether the age of attainment of competence to form specific γA antibodies parallels that of the other immunoglobulin classes. We have attempted, thus far unsuccessfully, to so adjust the dose of antigen within the lumen of the intestinal tract as to induce an antibody response restricted only to the lamina propria of the gut wall. The ability to do this might clarify the question of whether γA antibodies may be formed de novo or, as suggested by others, represent a sequential stage of differentiation of immunocytes forming antibodies of different classes.

The question of whether the immunologically competent lymphoid cell is pre-committed to the formation of antibodies of a restricted class, or alternatively constitutes a multipotential cell whose differentiation is controlled by local environmental factors has recently assumed great theoretical and practical significance in immunology. We have set up preliminary experiments to establish a model for the study of this question which show great promise. This involves surgical excision of a small portion of the ileum of the fetal lamb at a stage of gestation (prior to about 90 days) when lymphoid cells have not yet taken up residence in the gut wall. Anastomosis of the fetal ileum is then effected, so that the fetus may go to normal delivery without embarrassment. Pieces of the excised ileum in the area that would normally have progressed in the newborn to local antibody formation and even germinal center development are then placed into an ectopic location, in this instance into the anterior chamber of the eye through an incision in the cornea. Two of the three animals on which this procedure was performed went on to normal birth and showed that the model would work technically, since they presented the fascinating picture through the transparent cornea of a structurally normal piece of intestine growing in the anterior chamber, with even intestinal villi visibly floating in the aqueous humor. Since we know from previous experience that the induction of uveitis (an intraocular round cell inflammatory reaction) is accompanied by the local intraocular formation of only γM and γG antibodies within the iris, all of which immunologically active cells have come through the blood from outside the eye, we hope with specific fluorescent anti-immunoglobulins to be able to demonstrate that while γM and γG differentiation occurs in the normal ocular tissue, the immediately adjacent lamina propria of the implanted gut may cause the local differentiation of γA-forming cells.

B. The relationship between immunogenesis and lymphogenesis. We have, over the years, accumulated extensive histologic material from fetuses of all ages, either unstimulated or experimentally immunized by a variety of procedures and with a variety of antigens. We have recently gone through much of this material in an attempt to assess the relationship between the development of lymphoid tissues and structures in the fetus and its participation in active immunologic processes. The results of these studies are described in several recent publications (1-3), and several points of great interest emerged from these studies. The first point, which we have vaguely alluded to in earlier reports, is that frank lymphoid maturity in no sense precedes and paves the way for the attainment of immunologic competence: rather, the final stages of maturation of lymphoid tissue (i.e., the clearcut distinction between lymph node cortex and medulla, and the formation of germinal centers) appear to be as dependent upon exogenous stimulus as is the adoptive immune response. Lymphoid maturity, in fact, generally accompanies or even follows by a short lag the overt immune response to intrauterine immunization (1, 2).

The second point of interest to emerge from these morphologic studies was the fact that germinal center formation in the fetal lymphoid tissues appeared not to be a critical factor either in the development of the primary antibody response nor in the establishment of persisting immunologic memory. It will be recalled that the germinal center has variously been identified as a structure important in the focusing of antigen onto immunocytes in the immune response, in the retention of antigen to perpetuate the immune response, or as the seat of immunologic memory. In the present study, however, we found that while germinal center formation almost invariably accompanies immunization with complete Freund's adjuvants, that reasonable primary antibody responses and a preparation for secondary responses to intravenous antigen may not involve germinal center formation. Further, the relatively early and apparently spontaneous formation of germinal centers in the Peyer's patch region of the intestinal tract, earlier thought to represent a central lymphoid control tissue, has been shown in our studies to result from stimulization across the gut epithelium from the lumen, so that formation of a mid-ileal atresia in the fetal lamb led to suppression of Peyer's patch formation in the

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distal ileum, while increase in stimulus from the intestinal lumen by feeding the fetus resulted in a precocious maturation of these structures. These observations on the lack of critical importance of the germinal center in organized lymphoid tissue to the immune response led us to a broad examination of the significance of the formation of germinal centers in ectopic (extra-lymphoid) tissues. A study of a variety of chronic, mostly infectious processes (4) revealed that the germinal center may occur in almost any tissue or organ in the body where an appropriate chronic stimulus exists. While germinal center formation often but not invariably accompanies local antibody formation following an immunogenic stimulus, the converse is by no means true and local antibody formation may persist for long periods of time in extra-lymphoid locations without the accompanying formation of germinal centers. This suggests that the germinal center may not subserve a uniquely immunologic role, but may well be engendered by non-immunologic stimuli.

The third interesting aspect of this study is pertinent to the currently popular question in immunology of the basis for the generation of the broad range of diversity shown by antibody molecules. The major hypotheses in this area are either that the information for all antibody specificities are transmitted genetically through the germ line, or alternatively that only a few immunoglobulin chain backbones are included, and that the diversity is generated by some process of somatic mutation or recombination of DNA sequences. While one or another of the latter models appears most commonly accepted, the main body of ontogenetic data seems to point strongly toward the former suggestion (3). Thus any somatic model, requiring as it would the chance development of any particular antibody specificity during ontogeny, could hardly explain the data on the very precisely timed maturation of immunologic competences which we have observed, nor the normally broad range of heterogeneity of the early fetal responses with respect to immunoglobulin classes, affinities of antibodies, or ranges of cross-reactivities with other antigens. But perhaps most significant in this respect is the observation that the fetal lamb at some 40 days gestation can form antibodies to bacteriophage virus at a time when its total lymphoid numbers are so low (less than 10^5 - 10^6 lymphocytes) that sufficient numbers of lymphoid cell generations to satisfy any somatic model could hardly have developed within this small animal.

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C. The modulation of host-parasite relationships in the immunologically maturing fetus. We described in earlier reports the results of experimental infection of the fetal lamb at differing gestational ages with the bluetongue live virus vaccine, innocuous to the adult animal but which in the young fetus produces encephalopathy and retinal dysplasia (5). The latter observation led us into a broad histopathologic study of the various types of retinal dysplasia which present as congenital anomalies in the human infant, and of the pathogenetic mechanisms whereby this problem of organogenesis may arise (6). We have described in detail the pathology of the hydranencephaly and porencephaly induced in the fetal lamb by this live virus vaccine (7). In addition, we have worked up during the past year a description of the pathogenesis of this infectious process in the fetal lamb (8) which involves an apparent specific cytopathogenic effect of the virus on those cells in the developing fetal brain which migrate from the ependymal plate. This stage of apparently developmental susceptibility of cells of the central nervous system to this virus ends at approximately the same time that the fetus becomes immunologically competent to form circulating antibodies against these viral antigens, the appearance of which terminates the fetal viremia.

In an analogous series of experiments, we have found that lymphocytic choriomeningitis virus, responsible for a well-defined immunopathologic encephalopathy in the mouse, appears to produce in the young fetal lamb a non-immunologic cytopathogenic encephalopathy. As in the case of the bluetongue viral infection mentioned above, the virus appears to lose its pathogenic properties as the fetus continues to mature, and the onset of active antibody formation within the fetus leads to clearance of the virus from its circulation. Immunosuppression of the fetus accomplished

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by thymectomy followed by treatment with antilymphocyte serum results in inability of the fetus to form antibodies, a long-standing fetal viremia which lasts into the neonatal period, but no development of CNS pathology despite the high and persisting titer of virus in the brain. We hope to be able to establish whether recovery of the viremic immunosuppressed animal will lead to the development of an immunopathologic process analogous to that seen in the mouse, and to further clarify the manner in which the immunologic status of the host affects the outcome of its interaction with this and analogous pathogenic organisms. These studies are being performed in collaboration with Dr. Gerald Cole of The Johns Hopkins School of Hygiene and Public Health.

D. Other studies. In collaboration with Dr. William Paul of the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, we have attempted to ascertain whether the lymphoid cells of the developing fetus contain immunoglobulin receptors on their surfaces prior to and after antigenic stimulus. Preliminary evidence suggests that both γM and γG receptors may exist on at least a portion of the fetal lymphoid cells prior to stimulus, and that the number of such cells detectable after primary stimulation increases markedly. Technical difficulties have thus far prevented meaningful quantitation of these results, and have made it difficult to identify antigen-specific receptors on these lymphoid cells.

Other studies reported elsewhere have shown that a protein derived from the mononuclear cell found in the coelomic cavity of the invertebrate sea star Asterias forbesi acts like a number of the effector molecules active in vertebrate delayed hypersensitivity reactions, possessing even for vertebrates a macrophage migration inhibition function and the ability to stimulate a delayed hypersensitivity-like lesion in the vertebrate skin. We have used this purified substance in microgram amounts, both intraocularly and intracutaneously in the fetal lamb, in order to test its general ability to support this type of mononuclear inflammatory reaction, and have found the fetal response to be analogous in all respects to that shown by the adult.

Conclusions:

1. Preliminary evidence would suggest that the earliest antibody responses in the developing fetus may involve all of the immunoglobulin classes and subclasses which the species is capable of making, and that no phenotypic class restriction in heterogeneity is demonstrable. In the case of those immunoglobulins formed primarily in certain specialized tissues (such as γA in the various secretory organs), active response requires the presentation of antigen at the local site.

2. Maturation of organized lymphoid tissues appears not to be a necessary prerequisite to the attainment of immunologic competence by the developing fetus. Full lymphoid maturation, like manifestation of the active immune response, is ultimately dependent upon exogenous stimulation. Germinal center formation does not appear to be necessary to either the primary immune response, nor to the preparation of the host for a secondary booster response.

3. Using the model of lymphocytic choriomeningitis virus infection of the fetal lamb, it has been found that the immunologic status of the host affects the amount and distribution of virus in the host, as well as the type and degree of resulting pathology. Immunosuppression of the fetal host results in a persisting viremia which may be related to true immunologic tolerance.

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13. ABSTRACT

These investigations represent a broad study of the nature of the immunologic maturation in the mammalian fetus in utero, and of the factors which affect and control this development. It is based upon the establishment of techniques permitting extensive and repeated surgical access to the fetus during gestation, and takes advantage of the special agammaglobulinemic and antigen- and germ-free environment of the fetus during gestation, permitting fundamental studies of the basic mechanism of host response to immunization. It was found that the attainment of immunologic competence to a given antigen permits the fetus to form antibodies of all immunoglobulin classes and subclasses, suggesting that there are no phenotypic lags in immunocyte differentiation. Stimulation of γA immunoglobulin requires, however, presentation of the antigen at the appropriate local site of production of this immunoglobulin. It is clear from these studies that organization of lymphoid tissues and even development of germinal centers is not a necessary prerequisite for the development of appropriate immune responses to immunization by the developing fetus. The ontogenetic data on the stepwise development of immunologic maturity in the fetus, on the ability to obtain immune responses in the presence of extremely small numbers of lymphoid cells early in gestation, and on the lack of a recognizable phenotypic restriction of heterogeneity of the early antibody response would appear to argue strongly against the theoretical model that antibody diversity is generated during ontogeny by a process of somatic mutation or

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recombination. Finally, experimental infection of the normal and immunosuppressed fetal lambs with lymphocytic choriomeningitis virus promises to yield interesting data on the development of immunologic tolerance to viral pathogens, and on the manner in which the immunologic status of the developing host may modulate the pathognesis of an infectious disease process.

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Immunization						
Antibody response						
Immunogenesis						
Lymphogenesis						
Congenital disease						
Pathogenesis						
Thymus						
Gut-associated lymphoid tissue						
Viral disease						

AD________________

ANTIBODY FORMATION AND IMMUNITY (U)

ANNUAL PROGRESS REPORT

by

Jonathan W. Uhr, M.D.

September 1971

(For the period 1 June 1970 to 31 May 1971)

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SUMMARY

Cell Surface Immunoglobulin

The proteins on surfaces of living splenic lymphocytes from normal mice were iodinated enzymatically. Specific immunoprecipitation of radiolabeled surface Ig obtained from lysates of a small lymphocyte fraction indicated that approximately 2-3% of the acid-precipitable radioactivity from the cell surface is Ig. Ninety-five percent of the Ig was recovered from the cell surface in the form of an IgM monomer. Control experiments suggested that the monomer did not result from depolymerization of 19S IgM by the methods used to radiolabel and isolate the molecule. ^{3}H-tyrosine labeling of IgM produced by myeloma cells and radioiodination of IgM in solution gave the same ratios of μ:L radioactivity as radiolabeling of IgM on cells, indicating that the tyrosine residues of L and μ-chains of cell surface IgM are available to the lactoperoxidase during the iodination. This is consistent with the hypothesis that cell surface IgM is entirely on the outside of the plasma membrane presumably attached to it by the Fc fragment.

Immunoglobulin Synthesis and Secretion

A. Nonsecreted Ig. - The synthesis and intracellular transport of Ig was studied in an established line of Burkitt lymphoma cells. Subcellular fractionation experiments in pulse-labeled cells indicate that this nonsecreted Ig is formed on membrane bound polyribosomes, enters the cisternae of the RER, is transported to the Golgi and, thereafter, to the plasma membrane probably via vesicles. The mechanisms involved appear to be similar to those in plasma cells.

B. Secreted Ig. - The intracellular site of incorporation of ^{3}H-fucose, a terminal sugar, into Ig was studied in MOPC-167, a murine myeloma tumor. Chemical studies defined the incorporated radioactivity as ^{3}H-fucose which was almost exclusively associated with Ig. Electron-microscopic radioautography indicated that the Golgi complex is the major site of incorporation.

Regulation of Antibody Formation

It was found that the addition or removal of antibody from immunized mice by exchange transfusion resulted in an exagerated response by the immune system, i.e., decreased or increased rate of antibody formation, respectively. A model to explain this regulation of antibody formation by serum antibody was proposed. Its major feature is an equilibrium between antibody in the circulation and antibody in the form of immune complexes in an extracirculatory immunogen containing compartment.

Cell Surface Immunoglobulin

There is considerable evidence that the capacity of lymphocytes to bind antigen specifically is due to Ig on the cell surface. The methodologies used for such studies rely on the capacity of anti-Ig antibody to make effective contact with antigenic determinants of the receptor. Such contact depends upon the presence of determinants on portions of the receptor available to anti-Ig antibody in the medium and retention of antigenicity when the receptor is bound to the plasma membrane.

We have developed a new approach in which surface Ig can be studied by standard immuno- and biochemical techniques after its removal from the cell surface. This was accomplished by radioiodinating the surface of living splenic lymphocytes, using a lactoperoxidase, as has been done for red blood cells by Phillips and Morrison (1).

Our studies indicate that the surface is radioiodinated without labeling intracellular molecules. Thus, subcellular fractionation of iodinated cells indicated that over 97% of the acid-precipitable radioactivity and all of the immunoprecipitable radioactivity was associated with membranes. Additional experiments excluded iodination of intracellular protein by endogenous peroxidases or by the added lactoperoxidase.

In lymphocytes from spleens of normal mice, regardless of the subpopulation obtained from density gradients, 2-4% of the total radiolabeled material on the cell surface after release by detergent was specifically precipitable by anti-Ig. Experiments performed *in vivo* and *in vitro* provided no evidence that this Ig was adsorbed to the cells from the serum or medium. It is therefore concluded that the Ig, which presumably represents the antigen-specific receptor, was formed by the cells on which it resides. Identification of this Ig on acrylamide gels after immunoprecipitation with class-specific antiserum indicates that over 95% of the radioactive Ig from the small lymphocyte fraction is IgM (see Figure 1). The large lymphocytes and plasma cells also have the majority of radioactivity in IgM (90%) and only 10% in IgG. Therefore the small percentage of IgG molecules on small lymphocytes is probably not due to contamination with large lymphocytes and/or plasma cells. These results are in good agreement with previous studies of Warner *et al.* (2), in which IgM was shown to be the dominant antigen-specific receptor on mouse splenic lymphocytes, and with Pernis (3) who demonstrated that IgM is the major class of Ig on rabbit splenic lymphocytes.

Virtually all of the cell surface IgM was recovered under nonreducing conditions as monomer (Figure 2). Since monomers of mammalian IgM are held together by disulfide bonds, the above finding suggests that monomer is the form of the molecule on the cell surface. The possibility that the monomer is generated by self-assembly of H and L chains after detergent lysis of cells was excluded by the results of lysing cells in the presence of alkylating agents. Degradation of polymeric IgM by conditions in the lysate or by iodination, detergent treatment, or solubilization of immunoprecipitate was not supported by appropriate control experiments using 19S serum IgM (also shown in Figure 2) and mouse tumor (MOPC-104E) IgM.

The relationship of the IgM molecule to the plasma membrane has not yet been elucidated. The release of IgM monomer of the expected molecule weight by detergent lysis is presumptive evidence that the molecule is bound noncovalently to the plasma membrane. Of particular interest is the finding that the ratio μ:L chain radioactivity is similar in radioiodinated IgM from spleen cell surfaces or serum as well as ^{3}H-tyrosine-labeled monomeric IgM (MOPC-104E). These findings suggest that the tyrosine residues of μ and L chain are available to the lactoperoxidase even when the IgM is attached to the plasma and argue against a model in which a large portion of the Fc fragment is buried in the membrane. This supports our previous suggestion that the receptor molecule is entirely on the outside of the plasma membrane and is attached to it by its Fc fragment, i.e., Ig molecules destined for secretion or to act as receptors become bound to the inside of Golgi vesicles by the Fc fragments, are transported to the plasma membrane within such vesicles, and are exteriorized by reverse pinocytosis.

Studies with anti-θ and "pure" B and T cells show that the IgM molecules isolated in our studies come from the surface of B lymphocytes primarily. These cells are the precursors of antibody-secreting cells. In most immune responses, however, the vast majority of antibody-secreting cells (and serum antibody) is eventually IgG. Thus, the major possibility to explain our findings is that lymphocytes that have synthesized IgM "switch" to IgG synthesis after stimulation by antigen. This could occur in cells secreting IgM antibody as suggested by others (4-7) or in lymphocytes that have only synthesized receptor. Thus, IgG-secreting cells could have IgM receptors (8).

Immunoglobulin Synthesis and Secretion

The initial steps in the synthesis and intracellular transport of immunoglobulin (Ig) secreted by plasma cells are partially understood. The concept has emerged that Ig chains are synthesized by membrane-bound polyribosomes enter the cisternae of the rough endoplasmic reticulum (RER) and remain within membranes until their secretion from the cell.

It is well established, however, that lymphocytic cells which lack a well developed RER are also capable of Ig synthesis. Thus, mechanisms of synthesis and intracellular transport other than those described for plasma cells may operate in these cells. To investigate this possibility, we studies a human lymphoma cell line which synthesizes IgM but lacks a well developed RER. This cell line (Daudi) has been shown not to secrete its IgM (9).

Our experiments indicate that these mechanisms of Ig synthesis and intracellular transport are utilized in cells which have two characteristics strikingly different from plasma cells: a paucity of RER and a failure to secrete Ig. The experiments show that (a) Ig is synthesized by polyribosomes bound to the ER. Thus, when nascent chains were released from polyribosomes and precipitated with specific antisera to Ig, 3.3 ± 1.3% of the incorporated radioactivity in the bound polyribosomes and 0.4 ± 0.5% of the incorporated radioactivity in the free polyribosomes were precipitated as Ig. (b) Ig rapidly enters the microsomes, (c) Ig is presumably transported to the Golgi complex wherein galactose and fucose are added, and (d) Ig appears to remain associated with membranes throughout its intracellular life as judged by subcellular fractionation (Figures 3 and 4). These results, therefore, remove the impetus for suggesting novel mechanisms of synthesis and transport of Ig within lymphocytic cells and suggest that the above features may be general ones for cells synthesizing Ig.

At what point is intracellular transport of Ig terminated within Daudi cells? The biosynthetic studies described above imply that the block in intracellular transport does not occur at a pre-Golgi site. This conclusion together with the demonstration by Klein (9) that Daudi cells can specifically bind fluorescein labeled anti-Ig to their cell surface strongly suggests that Ig molecules are transported to the plasma membrane but are not released from the cell.

The inability of Daudi cells to secrete Ig could result from a defect in the secretory mechanism or it might be a characteristic feature of a certain class of lymphoma cells. Ig synthesis by Burkitt lymphoma cells has been studied extensively by others. The results indicate that at least 5 of 15 lines examined synthesize but do not secrete Ig. Approximately 2/3rd of the lines make μ chains.

There is additional evidence to suggest that the absence of secretion may be a characteristic of some lymphomas. First, murine lymphomas induced experimentally are typically nonsecretory, although intracellular Ig has been detected in some lines (10); in contrast nonsecretory variants which arise from myeloma lines usually do not synthesize Ig (10-13). Second, proliferative disorders of lymphoid tissue are rarely associated with the appearance of homogenous Ig in the serum or urine (14); however, the appearance of such proteins is considered pathognomonic for multiple myeloma (15).

These considerations and the high incidence of Ig-synthesizing, nonsecretory lymphoma lines suggest that these lines may have arisen from normal lymphocytes which do not secrete Ig. Since Daudi cells have a receptor for C', they are probably derived from B cells which are the precursors of antibody-secreting plasma cells. Also, μ chains are the predominant heavy chain on B cells. Thus, Burkitt lymphoma lines which do not secrete Ig and which show a high incidence of μ chains may be derived from B cells and their Ig may therefore be the counterpart of the antigen specific receptor.

Our previous studies with myeloma cells suggested a model for the synthesis and transport of Ig destined for secretion. The present studies with Daudi cells and the considerations discussed above suggest that this model can readily explain the genesis of the immunoglobulin which acts as antigen-specific receptor: (a) Ig is made on bound polyribosomes, enters the cisternae of the RER, and is transported to the Golgi complex; (b) Ig becomes bound by its Fc portion to the membrane of the Golgi vesicles; (c) a post-Golgi vesicle migrates to the plasma membrane and fuses with it; (d) by reverse pinocytosis, the Ig molecule becomes exteriorized with its Fab portions available for antigen binding and its Fc portion attached to the plasma membrane (formerly Golgi vesicle). This model is similar to the one proposed for secretory cells except that Ig is not released from the plasma membrane. The model can be tested by experiments designed to establish the intracellular life history of the Ig receptor and its relationship to the plasma membrane in normal lymphocytic cells.

In previous studies with murine myeloma cells, it has been shown that sugars are incorporated into immunoglobulin (Ig) at two sites: The "bridge" sugar, N-acetylglucosamine is incorporated into nascent chains on polyribosomes, and additional glucosamine residues are incorporated onto completed chains, probably in the Golgi complex. Galactose, a sugar that occurs more distal in the carbohydrate moiety, is incorporated in the Golgi complex in regard to the terminal sugars of the carbohydrate moiety, Choi et al. (16) have reported that the fucose content of light (L) chain in a subcellular fraction containing smooth endoplasmic reticulum (SER) is about one-fourth of that found in urinary L chain whereas galactose content in the SER fraction is approximately half of that found in L chain. They interpret this finding as suggesting that fucose may be added at a later stage and at a different site than galactose. Swenson and Kern (17) have shown that the kinetics of incorporation of sialic acid, another terminal sugar, into intracellular and secreted Ig is identical suggesting that sialic acid is added immediately prior to secretion of Ig. These findings suggest that the terminal sugars of Ig could be added at a third site "distal" to the Golgi complex, perhaps the plasma membrane.

In the present studies, we have investigated the site of fucose incorporation in murine myeloma cells. It was shown by appropriate chromotographic and biochemical procedures of labeling employed, ^{3}H-fucose is incorporated as such into Ig and that no significant incorporation occurs into other macromolecules in the cytoplasm. It was therefore possible to interpret electronmicroscopic radioautographs of labeled myeloma cells in terms of Ig synthesis.

The results indicate that the Golgi complex is the main if not the exclusive site of incorporation of fucose into Ig. Thus, at the earliest time point (15 min) that significant numbers of grains could be visualized on the radioautograms (which should reflect primarily incorporation rather than incorporation and transport) the majority of grains were over the Golgi complex and virtually none was seen over the plasma membrane. The results with fucose appear strictly analogous with those we have reported with galactose using a murine myeloma tumor that secretes IgG.

Regulation of Antibody Formation

In previous experiments, serum antibody to bacteriophage was lowered by exchange transfusion. Following the removal, there was a marked rise in antibody levels which frequently reached peak titres exceeding those prior to removal. The assay used in these studies was phage neutralization which is affected by the binding affinity of antibody. Therefore, the possibility was considered that the level of antibody observed at the peak of the rise that followed removal might be due in part to a change in binding affinity.

It appeared important, therefore, to repeat these experiments using an antibody assay that would allow estimation in molar terms (anti-Keyhole limpet hemocyanin (KLH)). In the experiments to be described, antibody levels to KLH and T_2 in mice have been specifically increased or decreased by exchange transfusion, and the resultant antibody levels to KLH measured quantitatively by coprecipitation under conditions of extreme antigen excess.

Figure 5 shows the average antibody response to KLH (5A) and T_2 (5B) in groups of mice in which exchange transfusion was performed 30 days after immunization. The recipient mice were immunized to KLH and T_2 and the donors of the blood to one antigen only. Since there is antigenic competition between the two antigens, the donors had higher antibody levels to the antigen in question. Thus, the exchange lowered levels to one antibody and raised levels of the other. Pre-exchange titre is used as a reference point, and antibody levels on subsequent days are plotted as a percent of that value.

As can be seen in Figure 5A, in the control group anti-KLH levels increased slowly during the period of the experiment. In the group in which antibody levels were depleted by 65%, there followed a rapid and prolonged rise in titres so that 10 days later the concentration of anti-KLH molecules in the circulation was approximately 20% higher than that of the control group. In contrast, the addition of anti-KLH antibody which resulted in an immediate increase of 150% in levels, was followed by a rapid decrease so that 10 days later, the antibody level was 50% below the control group.

Figure 5B shows the same three groups of animals titered for antibody to T_2. The group depleted of anti-T_2 is the same group that received additional anti-KLH, and the group that received added anti-T_2 is the same group in which anti-KLH was depleted. The results with anti-T_2 are analogous to those described for anti-KLH.

These results suggest that elevation of the serum level of a given antibody results in decreased synthesis of that antibody and that depletion results in its increased synthesis as manifested by a rise that not only repletes the "lost" antibody but exceeds the expected (control) levels. The regulation is specific as demonstrated by the above experiments in which a single animal immunized to two antigens had antibody of one specificity depleted and antibody of the other specificity added; subsequently, opposite effects on antibody levels to the two antigens were observed.

It is also of interest that despite the early effects of altering antibody levels on antibody formation, over the following month, the levels of all three groups (control and experimentals) approached one another (particularly evident in the anti-T_2 responses). This could be considered further evidence for an effective regulatory mechanism.

The above experiments suggest that under physiological conditions the level of serum antibody can regulate antibody synthesis. Hence, it can be postulated that a feedback mechanism exists whereby serum antibody levels modulate the antigenic stimulation necessary for antibody synthesis.

A system analysis of this problem suggests the following simple model of feedback regulation of antibody synthesis by circulating antibody (Figure 6). Its major features are:

(a) An extracellular immunogen-containing compartment in which a portion of the antigen which is not immediately catabolized persists for a prolonged period of time. Multiple dynamic equilibria are postulated between free antigen, free antibody, and antigen-antibody complexes of various ratios. Inherent in this concept is that although the equilibria favor complex formation, free antigen and immunogenic complexes must be present. (The immunogenicity of complexes is inversely related to antibody-antigen ratio.)

(b) An equilibrium between circulating antibody and antibody in the immunogen-containing compartment. Alterations in serum antibody level will eventually be reflected by changes in concentration of antibody in the immunogen-containing compartment. Depending on the direction of the change, antibody-antigen complexes will form or dissociate, in accordance with the mass-law principle. Thus, reduction in serum antibody will promote dissociation of complexes toward free antibody, and concommitantly increase free antigen and complexes of lower antibody-antigen ratio. The greater availability of free antigen and immunogenic complexes will increase stimulation of immune cells (as depicted by the heavier arrows in Figure 6), and lead to a new burst of antibody synthesis. Obversely, increase in serum antibody levels will promote complex formation, thereby depleting free antigen and decreasing stimulation of antibody synthesis

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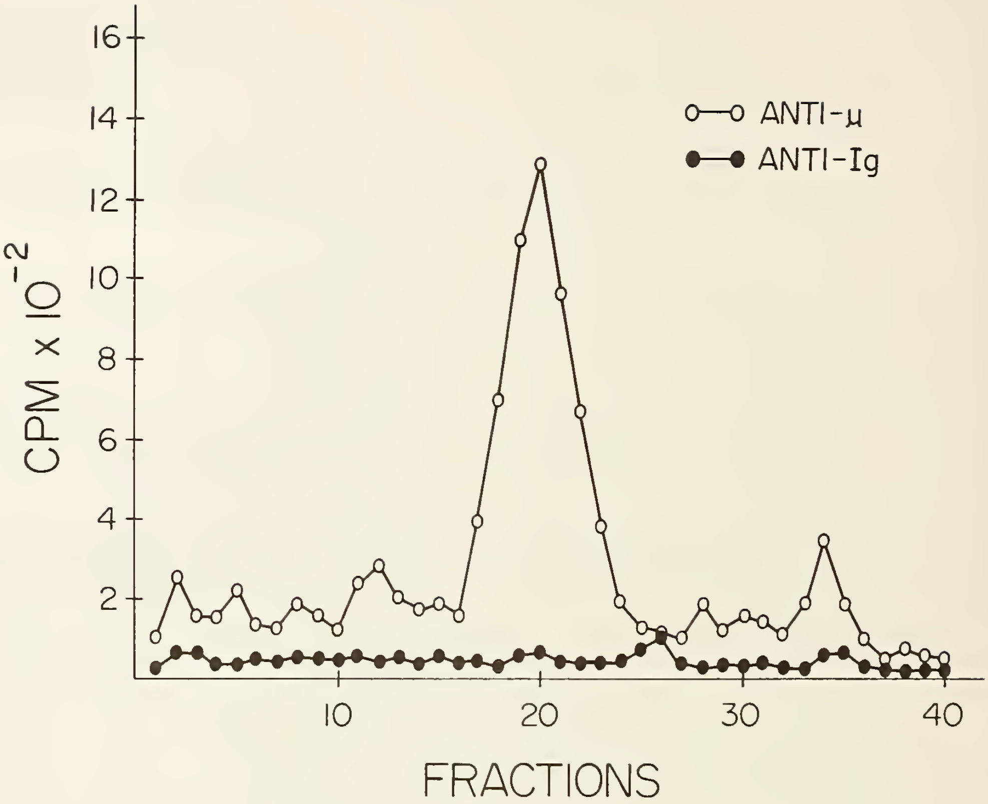

Figure 1. Comparative amounts of radioactive IgM and other classes of Ig from the surface of small splenic lymphocytes. After precipitation of IgM only, the supernatant was treated with anti-Ig and immunoprecipitated by a sandwich technique. Precipitates were reduced and alkylated before electrophoresis on acrylamide gels.

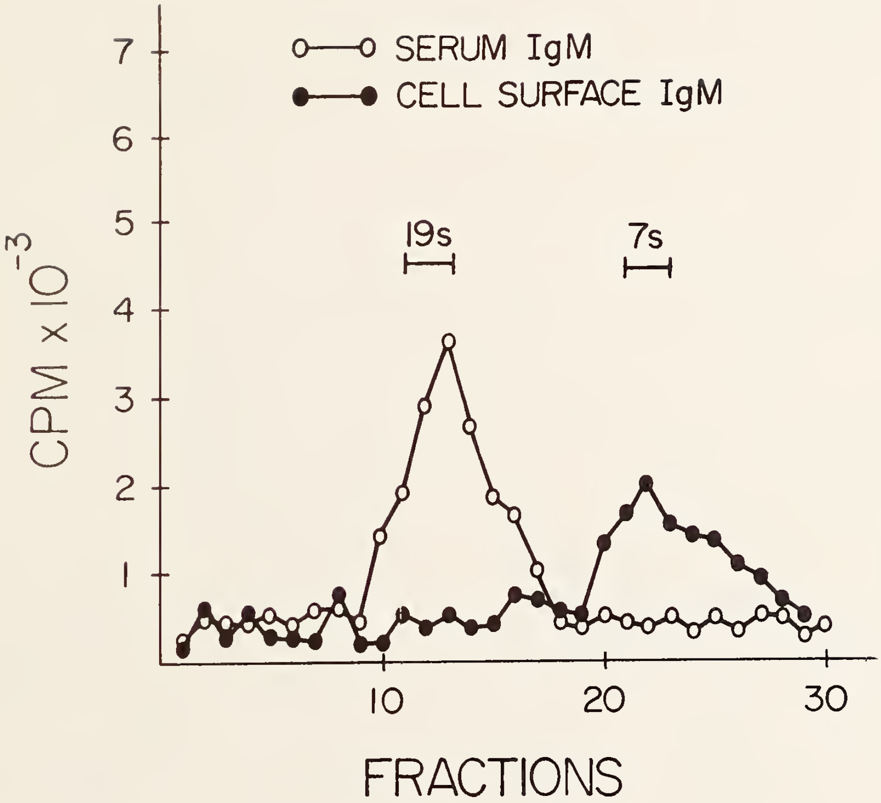

Figure 2. - Sedimentation in sucrose gradients of radiolabeled IgM from serum of hyperimmunized mice and from surface of splenic lymphocytes. Linear gradients containing 5-20% sucrose in 1% SDS and 0.5 M urea were employed. A control tube contained radioiodinated human IgM and mouse IgG as markers.

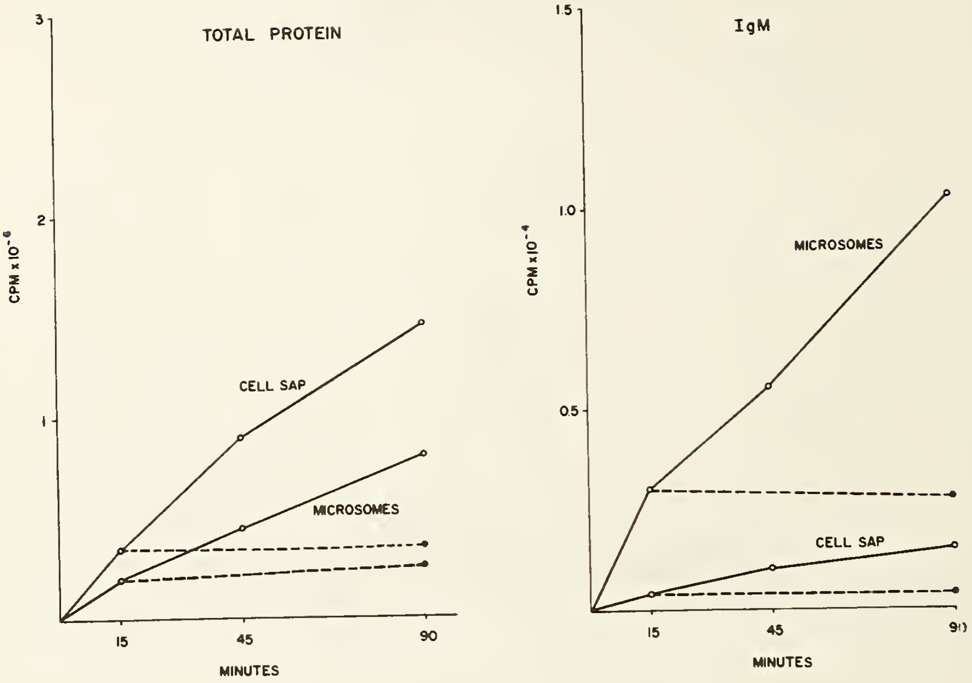

Figure 3. - Incorporation of ^{3}H-leucine into total protein and Ig in the microsomes and cell sap of Daudi cells. Cells were labeled for various times, microsomes and cell sap were separated, and radioactivity in acid precipitates and immunoprecipitates was determined. 0——0 continuous labeling, 0----● 15 min labeling, 75 min chase.

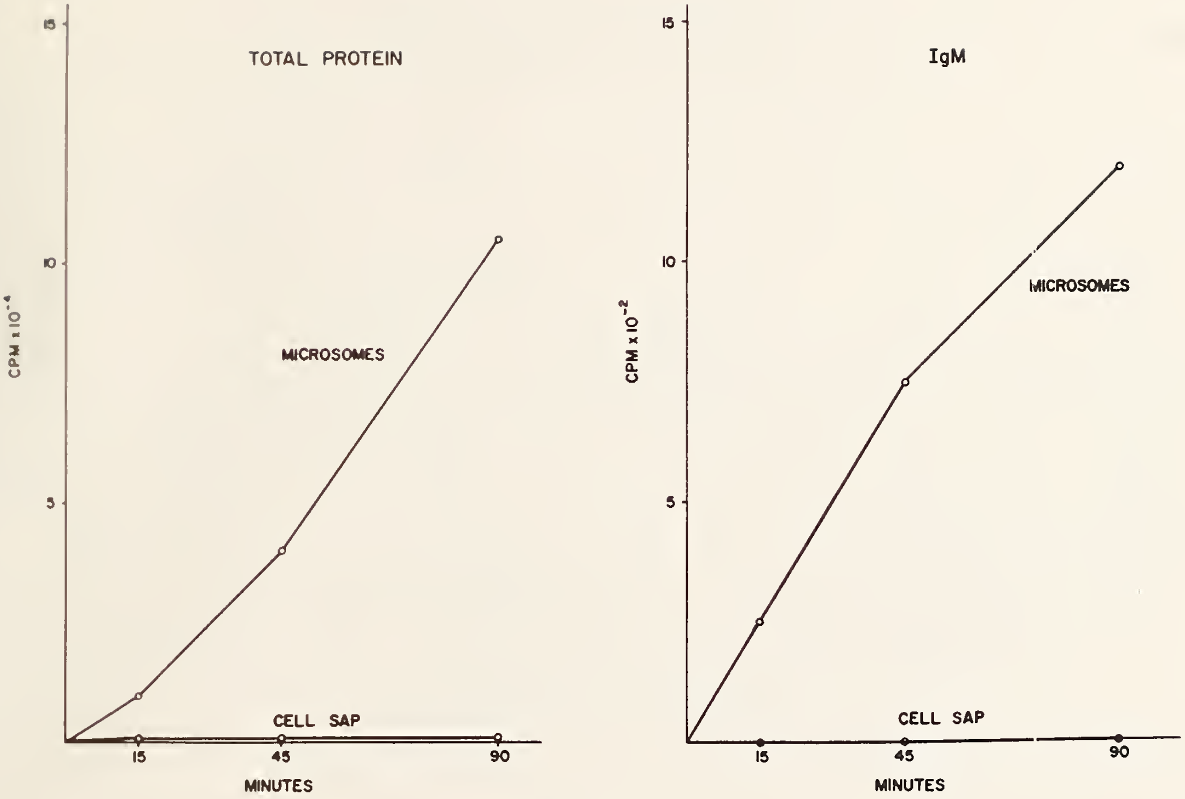

Figure 4. - Incorporation of ^{3}H-galactose into total protein and Ig in the microsomes and cell sap of Daudi cells. (See Figure 3)

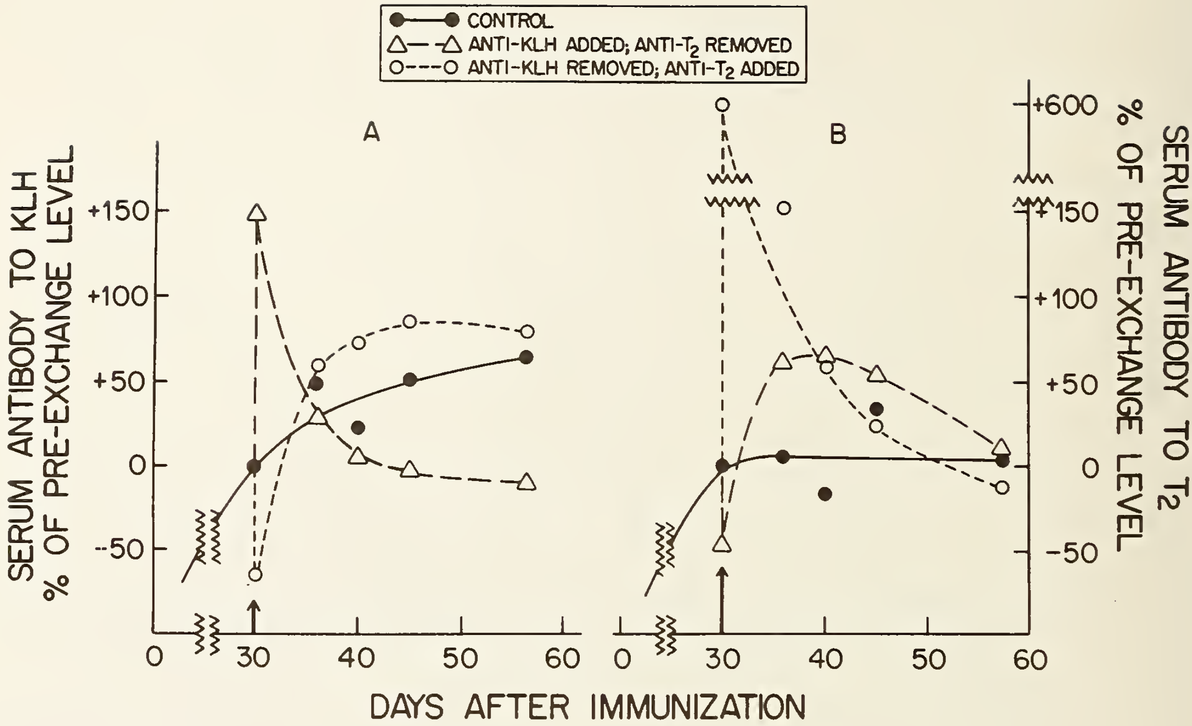

Figure 5. - The effect of alterations of serum antibody levels on the primary antibody response to KLH (Fig. A) and T_2 (Fig. B) in mice. Fifteen mice were immunized to both antigens 30 days previously. In one experimental group of five animals, serum level of anti-KLH was lowered and anti-T_2 raised by performing exchange transfusions (↑). In another group of five animals, anti-KLH was raised and anti-T_2 lowered.

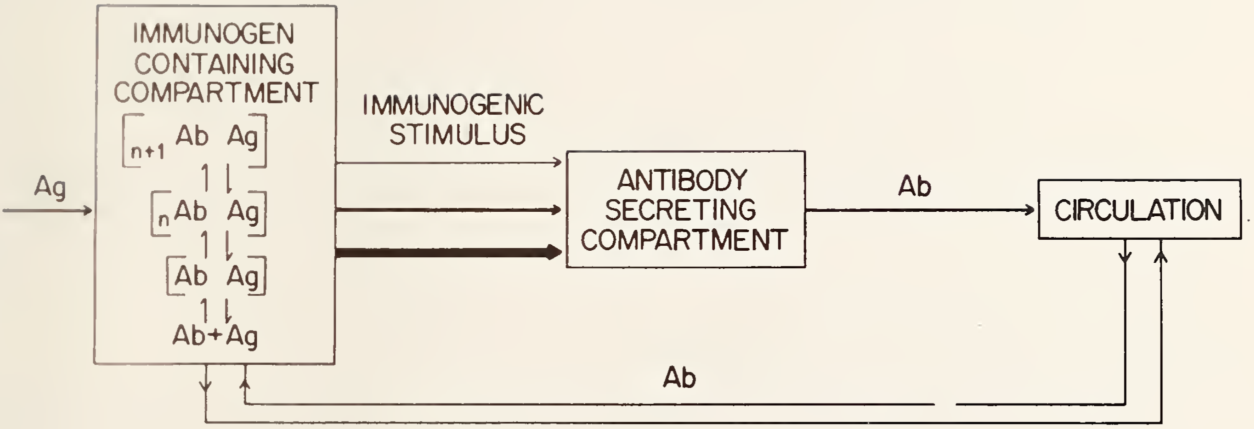

Figure 6. - A model of the regulation of specific antibody synthesis by serum antibody.

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13. ABSTRACT

Cell Surface Immunoglobulin. The proteins on surfaces of living splenic lymphocytes from normal mice were iodinated enzymatically. Specific immunoprecipitation of radiolabeled surface Ig obtained from lysates of a small lymphocyte fraction indicated that approximately 2-3% of the acid-precipitable radioactivity from the cell surface is Ig. Ninety-five percent of the Ig was recovered from the cell surface in the form of an IgM monomer. Control experiments suggested that the monomer did not result from depolymerization of 19S IgM by the methods used to radiolabel and isolate the molecule. ^{3}H-tyrosine labeling of IgM produced by myeloma cells and radioiodination of IgM in solution gave the same ratios of µ:L radioactivity as radiolabeling of IgM on cells, indicating that the tyrosine residues of L and µ-chains of cell surface IgM are available to the lactoperoxidase during the iodination. This is consistent with the hypothesis that cell surface IgM is entirely on the outside of the plasma membrane presumably attached to it by the Fc fragment.

Immunoglobulin Synthesis and Secretion. A. Nonsecreted Ig. - The synthesis and intracellular transport of Ig was studied in an established line of Burkitt lymphoma cells. Subcellular fractionation experiments in pulse-labeled cells indicate that this nonsecreted Ig is formed on membrane bound polyribosomes, enters the cisternae of the RER, is transported to the Golgi and, thereafter, to the plasma membrane probably via vesicles. The mechanisms involved appear to be similar to those in plasma cells.
B. Secreted Ig. - The intracellular site of incorporation of ^{3}H-fucose, a terminal sugar, into Ig was studied in MOPC-167, a murine myeloma tumor. Chemical studies defined the incorporated radioactivity as ^{3}H-fucose which was almost exclusively

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(Continued from previous page)

associated with Ig. Electronmicroscopic radioautography indicated that the Golgi complex is the major site of incorporation.
Regulation of Antibody Formation. It was found that the addition or removal of antibody from immunized mice by exchange transfusion resulted in an exagerated response by the immune system, i.e., decreased or increased rate of antibody formation, respectively. A model to explain this regulation of antibody formation by serum antibody was proposed. Its major feature is an equilibrium between antibody in the circulation and antibody in the form of immune complexes in an extracirculatory immunogen containing compartment.

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RESPONSES OF HUMANS TO CHOLERA VACCINE IMMUNIZATION (U)

ANNUAL PROGRESS REPORT

by

W. F. Verwey, Sc.D.

September, 1971

(For the period 1 August 1970 to 31 July 1971)

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Office of the Surgeon General, Washington, D.C. 20314
in cooperation with the Commission on Immunization
of the Armed Forces Epidemiological Board

Contract No. DADA 17-68-C-8152
The University of Texas Medical Branch
Galveston, Texas 77550

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FOREWORD

It is a pleasure to give grateful recognition to the cooperation and assistance of Dr. G. J. Beto, Dr. M. D. Hanson, Mr. S. A. Lanier, Mr. T. D. Hutto, and Mr. W. D. Kutach of the Texas Department of Corrections. We wish to acknowledge especially the helpfulness of Captain C. C. Aldridge and Captain H. Cottrell who are in charge of the hospital unit where all of the work with the volunteers was done.

A variety of professional skills is required for the successful completion of research of this type. The participation of the following persons within The University of Texas Medical Branch is particularly acknowledged:

Medical Supervision - Dr. J. C. Guckian

Laboratory Immunology - Dr. Y. Watanabe
Mr. H. R. Williams, Jr.

Computation and Clerical Administration - Personnel of The University of Texas Medical Branch Research Computation Center; Mrs. M. Hannigan and Miss Susan Smith.

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

These experiments were carried on under the Guidelines on Human Research established by The University of Texas Medical Branch. These guidelines have been accepted by the U. S. Public Health Service.

SUMMARY

This study of the vibriocidal response to initial immunization and reimmunization with cholera vaccine employed four groups of volunteers, three of which have been immunized with two doses 4 weeks apart and one which received two weekly doses. The three groups have been reimmunized at intervals of 6 months, 1 year and 2 years; and the group immunized at the 1 week interval was reimmunized at 2 years. While further analyses are in progress, the following conclusions are available:

1. Initial immunization with two doses given 1 week apart produces significantly higher peak geometric mean vibriocidal titers than does initial immunization at 4 week intervals. In addition, there appears to be a decrease in the frequency of "poor responders" when the 1 week interval is used. However, these differences are not great and the current recommendation of the Armed Forces permitting an interval of 1-4 weeks between doses for initial immunization appears to be justified.

2. When individuals are reimmunized at 1 year and 2 year intervals, their serological response to immunization is at least as good as that attained by persons who were reimmunized every 6 months.

3. When initial immunization is performed with 2 doses of ordinary vaccine and when reimmunization is carried out with 0.5 ml of similar vaccine, the serological responses that resulted were not materially different from those obtained with volunteers using an Ogawa monovalent vaccine that has been proven to be significantly protective against Inaba and Ogawa cholera epidemics in East Pakistan and the Philippines, respectively.

4. In consideration of the above results, the fact that striking progress has been made in the therapy of cholera, and that cholera vaccination is no longer required for entry into the United States from "a cholera area", it is suggested that the policies of the Armed Forces relating to cholera immunization be reviewed.

INTRODUCTION

In previous studies on the responses of human volunteers to cholera vaccine immunization carried on under Contract DADA 17-67-C-7084, groups of at least 17 human volunteers were used to study the effects of antigen quantity and the number and spacing of doses upon response to initial immunization. In addition, the effects of dosage size and number on responses to reimmunization were studied. The criterion of response to immunization was the tube dilution vibriocidal assay. Correlation between vibriocidal antibody levels and attack rates of naturally-acquired cholera have been reported by Mosley (1) for Inaba-type cholera in unimmunized persons, vaccine immunized populations, and familial contacts of cholera cases. In addition, very recently in our own laboratory, protection of rabbits against ileal loop challenge with vibrio cholera Inaba serotype has been found to be fairly well correlated with circulating vibriocidal antibody titers in actively immunized animals. It would therefore appear that this test may be a relatively satisfactory indication of the state of immunity produced by cholera vaccine immunization.

In our previous work under the above numbered contract, several observations were made which could have implications in relation to cholera vaccine immunization practices and appeared to require either confirmation or extension.

A. At the time this work was initiated the currently recommended immunization procedure in the Armed Forces was the use of a 0.5 ml dose followed in approximately 4 weeks by a 1.0 ml dose. Our studies indicated that in most instances the response to the second dose was less than to the initial dose. A booster effect was definitely not observed.

B. The same two doses administered one week apart appeared to produce higher geometric mean titers and a decrease in the frequency of "poor responders". Although this regime was numerically superior at all points in the immunization curve, the differences did not achieve statistical significance with the number of volunteers that were used in the initial study.

C. On reimmunization at 6 months, 0.5 ml of cholera vaccine produced maximum geometric mean vibriocidal titers that were significantly lower than those found on initial immunization. In addition there appeared to be an inverse correlation between the height of the initial response and the height of the response on reimmunization.

The work undertaken under the present contract has been designed a) to investigate further the relative effectiveness of two doses of cholera vaccine 1 week apart, and two doses 4 weeks apart, using larger numbers of volunteers; and b) to investigate the response of individuals to reimmunization at 6-month intervals compared to the response to reimmunization at 1 year and 2 year intervals.

MATERIALS AND METHODS

Volunteers. The human volunteers used in this study were inmates of the Ramsey Unit of the Texas Department of Corrections, and were males between the ages of 21 and 35 years. Preimmunization bleedings were obtained from each individual, and the various groups that were created in this study were balanced in terms of the heights and frequencies of preimmunization vibriocidal antibody titers. The groups were similar in respect to race, height, weight, age and the frequency of military service and travel outside of the United States.

Vaccine. In this study the same commercial vaccine used in the previous studies was employed for initial immunization, and the first reimmunization at 6 months. This vaccine was designated "CV" and in mouse protection tests was found to have a protective potency approximately equivalent to the Ogawa and Inaba NIH reference vaccines. It would therefore be considered to be a "minimal satisfactory vaccine". A second lot of vaccine was required for the reimmunization portion of this study beyond the first 6 months, and, by good fortune, this vaccine designated "CV2" had a mouse protection potency very similar to the first lot. All vaccine supplies were held continuously under refrigeration at 2-5°C during this study.

Plan of Study. Table 1 is a Flow Chart demonstrating the subdivision of the initial pool of volunteers into groups and indicates the injections and bleedings that each has received. This protocol has now been completed. Group 1 received initial immunization consisting of two doses 4 weeks apart, with reimmunization at 6-month intervals. This represents currently recommended immunization procedure. Groups 2 and 3 received similar initial immunization, but these groups were reimmunized after 1 year and 2 years respectively. Group 4 received initial immunization with two doses 1 week apart, and were reimmunized only at the end of 2 years. From the protocol, it is apparent that for certain types of analyses these groups can be combined to provide the statistical advantages of larger numbers; for instance, Groups 1, 2, and 3 are identical through the first 6 months and Groups 2 and 4 are the same through the whole first year of the study.

The Inclusion of Volunteer Groups from Previous Studies. Our previous work on cholera vaccine immunization under Contract DADA 17-67-C-7084 contained groups of human volunteers that had been immunized with the same dosage of the same vaccine and, for the first 6 months at least, were bled on the same schedule that has been employed in this study that was initiated almost 2 years later. Examination of comparable groups in the two studies has shown practically identical geometric mean vibriocidal titers at all time intervals. It has therefore been considered to be valid to combine these groups to obtain the advantages of increased numbers in certain statistical comparisons.

In consideration of this procedure it should be stated additionally that in respect to these two studies the same volunteer specifications were applied in the same prison and injections and bleedings were done in the same manner. All vibriocidal assays of both studies were performed by the same technicians using the same equipment and the same reference serum. As far as can be determined, the only variable between the two groups is time.

While these studies which we refer to as the H3 and H5 studies were in progress, other very similar work was being done with vaccines and purified antigens that were subsequently employed in epidemiological field trials in East Pakistan. Unlike the H5 studies and certain groups of H3, these vaccines were injected as a single 0.5 ml dose and the bleeding schedule was slightly different. One of these vaccines that was evaluated serologically was the Inaba monovalent vaccine which has subsequently been demonstrated to be highly protective against an Inaba epidemic in East Pakistan, and significantly protective against an Ogawa epidemic in the Philippines. Serological data obtained with a group of 31 volunteers receiving this vaccine has been included in this report because it offers a unique opportunity to relate the serological responses of the H3 and H5 studies to those obtained with a vaccine that has been epidemiologically proven to be effective against both Inaba and Ogawa type cholera.

Volunteers Removed from the Study. Losses from a study of this type are unavoidable, primarily due to transfer and unexpected parole. However, since the volunteers selected for this study were drawn from prison inmates having relatively long sentences or histories not conducive to parole, this rather long study has ended with numbers in each group that appear to be quite satisfactory. Table 2 gives the initial number of volunteers in each group and the number in the groups that completed the study. Although 41 persons were initially assigned into each group, one person in Group 2 was eliminated from the study before a significant amount of work had been done, and two others were moved to Group 3 at the end of the first year because they were temporarily unavailable for the required Group 2 immunization. Over the whole study, 86 of 164 persons (52%) remained in the study up to its termination.

Vibriocidal Antibody Titrations. Vibriocidal antibody titrations have been performed by a tube dilution method similar to that described by McIntyre and Feeley (2). Vibrio cholerae strains V86 and 041 have been the Inaba and Ogawa test strains that have been used. A reference serum tested in duplicate is included in each day's testing, and with the exception of the preimmunization bleeding titrations, 4 to 8 consecutive sera from each volunteer have been assayed on the same day and a proportional number of volunteers from each group are included in each day's testing. As in our previous studies, a standard series of serum dilutions was established, beginning at 1:2.5; therefore, all results can be expressed in terms of tube numbers, which then represent a progression based upon a dilution logarithm to the base of 2. Geometric means for the various groups can then be calculated using endpoint tube numbers in direct arithmetic computations.

Statistical and Computational Procedures. Vibriocidal titers are recorded on punch cards which serve as the input for an IBM 1800 computer which performed most of the statistical procedures that have been used in the analysis of these data. All geometric mean vibrioidal titers calculated in this study were accompanied by the simultaneous computation of the standard deviation and standard error for the groups.

Differences between means were tested for significance by the application of student's *t*-test and were considered to be significant if values for P were equal to or less than 0.05.

RESULTS

Figure 1 demonstrates the geometric mean vibriocidal antibody titers that were obtained with Groups 1, 2 and 3 during the full two years of this study. These groups all received initial immunization with two doses of vaccine 4 weeks apart. Group 1 was reimmunized every 6 months whereas Groups 2 and 3 were reimmunized at 1 year and 2 years respectively. These data are derived only from the volunteers who completed the study and therefore the mean titers represent data obtained from bleedings of the same persons.

The peak titers obtained from initial immunization and reimmunization of Group 1 are not significantly different. It is concluded that on a group-mean basis, there is no evidence of either a booster effect or a progressive depression of response associated with cholera immunization repeated at 6-month intervals over 2 years.

With five separate response curves for each individual, it is difficult to express individual patterns in numerical terms, but since each person represents approximately 5% of the group that was studied, the responses of individuals may have some importance. While about one-half of the group responded well and consistently to each immunization, one individual after reaching an Ogawa titer of 1:20,480 on initial immunization, never exceeded a titer of 1:1280 for either Ogawa or Inaba in the four successive reimmunization periods. There was a distinct tendency for good responses to be alternated with poor responses. Since these occurrences were not in phase with each other, the geometric means show much more stability than do the responses of indiviuals in the group. There was also a marked tendency for an unusually good response to one serotype to be accompanied by an unusually poor response to the other. Groups 2, 3 and 4 which received immunization at longer intervals, did not show this tendency for alternation of responses as observed with the members of Group 1.

Upon reimmunization at 1 year and 2 year intervals (Groups 2 and 3) the peak mean titers achieved were all numerically greater than those for Group 1 at the same period. These differences were not statistically significant, but clearly indicate that repeated immunization at 6-month intervals is not necessary to assure a satisfactory response to cholera vaccine.

Figure 2 shows the serological responses of volunteers who were initially immunized with two doses of cholera vaccine at 1 week and 1 month intervals and subsequently reimmunized at the end of 2 years. Although, as previously reported, the peak mean response to two doses a week apart is significantly superior to that obtained with two doses 4 weeks apart, and the frequency of low titers is diminished, both groups responded equally to reimmunization at the end of two years.

Figure 3 shows the response to initial immunization of all persons who received "CV" vaccine in both the previous study and the present one. This represents 159 volunteers who were given two doses 4 weeks apart, and 60 who received two doses 1 week apart. These geometric means are compared with 31 persons who had been given a single 0.5 ml dose of Inaba monovalent vaccine which has been used successfuly in field trials in East Pakistan and the Philippines. While the anti-Inaba titers of the volunteers receiving vaccine at 4-week intervals are significantly below those of the Inaba vaccine group for the first 3 weeks, the responses are very similar for the remainder of the 6 month period. The anti-Ogawa responses of persons in these studies are significantly superior to those produced by the Inaba monovalent vaccine. It would therefore appear that, to the extent that vibriocidal titers may be considered as an indication of immunity, standard vaccines used for initial immunization in two doses are producing responses equivalent or superior to those achieved in the same type of volunteer with a single dose of a provably potent monovalent vaccine.

DISCUSSION

This study was initially undertaken to investigate the effects of intervals between doses on initial immunization and the response of volunteers to various intervals between reimmunizations. It has shown a slight but consistent superiority for initial immunization given as two doses a week apart both in terms of peak mean vibriocidal titers and, as discussed more extensively in previous reports, a decrease in the frequency of "poor responders". In consideration of the relatively small differences that have been found, however, it would appear that the latitude that is allowable under the present Armed Forces recommendations permitting reimmunization at 1 week to 4 week intervals is justified; and the selection of dosage interval for initial immunization can be made on factors other than the effectiveness of the immunization procedure.

In regard to the interval between initial immunization and reimmunization, these results indicate that immunization at 6-month intervals is certainly not necessary to ensure a satisfactory reimmunization response, and if anything, reimmunization responses may be slightly better if the 1 year or 2 year interval is used. In assessing the immune status of individuals during the period from 6 months to 2 years, it should be considered that all geometric mean titers up to 2 years have remained above the mean vibriocidal level that Mosley, _et al_. have associated with significant immunity in the East Pakistan studies. It should also be considered that current studies with Inaba monovalent vaccine in East Pakistan are showing significant protection of children who were not reimmunized prior to a second yearly epidemic of Inaba cholera.* This current result in East Pakistan appears to be in support of the observations of Benenson, _et al._, who reported significant immunity occurring in adults in East Pakistan during the second year after immunization. Since immunization within the previous 6 months is no longer a requirement for reentry into the United States, and great strides have been made in the therapy of cholera over the last several years, it is suggested that practices relating to cholera immunization in the Armed Forces be reconsidered.

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1. Mosley, W.H. 1969. Texas Reports on Biology and Medicine _27_: Supplement 1, 227-241.

2. McIntyre, O.R. and Feeley, J.C. 1964. J. Infect. Dis. _114_: 468-475.

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* Reported at the 25 June 1971 meeting of the NIH Cholera Advisory Committee

TABLE 1

Flow Chart of the Distribution of Volunteers into Groups and the Injection and Bleeding Schedule for the Groups

Group Designation			1	2	3	4	Bleeding Designation
Number of Volunteers			41	41	41	41	
Date	Project Time						
	Year	Week					
9/28/68	--	-2	B + E	B + E	B + E	B + E	B0
10/12/68	1	0	I	I	I	I	
10/19/68	1	1	B	B	B	B + I	B1
10/26/68	1	2	B	B	B	B	B2
11/2/68	1	3	B	B	B	B	B3
11/9/68	1	4	B + I	B + I	B + I	B	B4
11/16/68	1	5	B	B	B	B	B5
11/23/68	1	6	B	B	B	B	B6
11/30/68	1	7				B	B7
12/21/68	1	10	B	B	B	B	B8
4/12/69	1	26	B + I	B	B	B	B9
4/19/69	1	27	B				B10
4/26/69	1	28	B				B11
5/3/69	1	29	B				B12
5/24/69	1	32	B				B13
10/11/69	1	52	B + I	B + I	B	B	B14
10/18/69	2	1	B	B			B15
10/25/69	2	2	B	B			B16
11/1/69	2	3	B	B			B17
11/22/69	2	6	B	B			B18
4/11/70	2	26	B + I	B	B	B	B19
4/18/70	2	27	B				B20
4/25/70	2	28	B				B21
5/2/70	2	29	B				B22
5/23/70	2	32	B				B23
10/10/70	2	52	B + I	B + I	B + I	B + I	B24
10/17/70	3	1	B	B	B	B	B25
10/24/70	3	2	B	B	B	B	B26
10/31/70	3	3	B	B	B	B	B27
11/21/70	3	6	B	B	B	B	B28

B = Bleeding

E = Examination

I = Vaccine Injection
(All 0.5 ml except at
0:1 week and 0:4 week)

TABLE 2

The Number of Volunteers in Each Group Initially and at the Termination of the Study

Volunteers	Group 1	Group 2	Group 3	Group 4
Initial Number	41	38	43	41
Final Number	19	19	23	25
% Completions	46	50	53	61

FIGURE 1

GEOMETRIC MEAN VIBRIOCIDAL TITERS OF HUMAN VOLUNTEERS IMMUNIZED AT INTERVALS OF SIX MONTHS, ONE YEAR AND TWO YEARS

(ALL INITIAL IMMUNIZATION WAS TWO DOSES FOUR WEEKS APART)

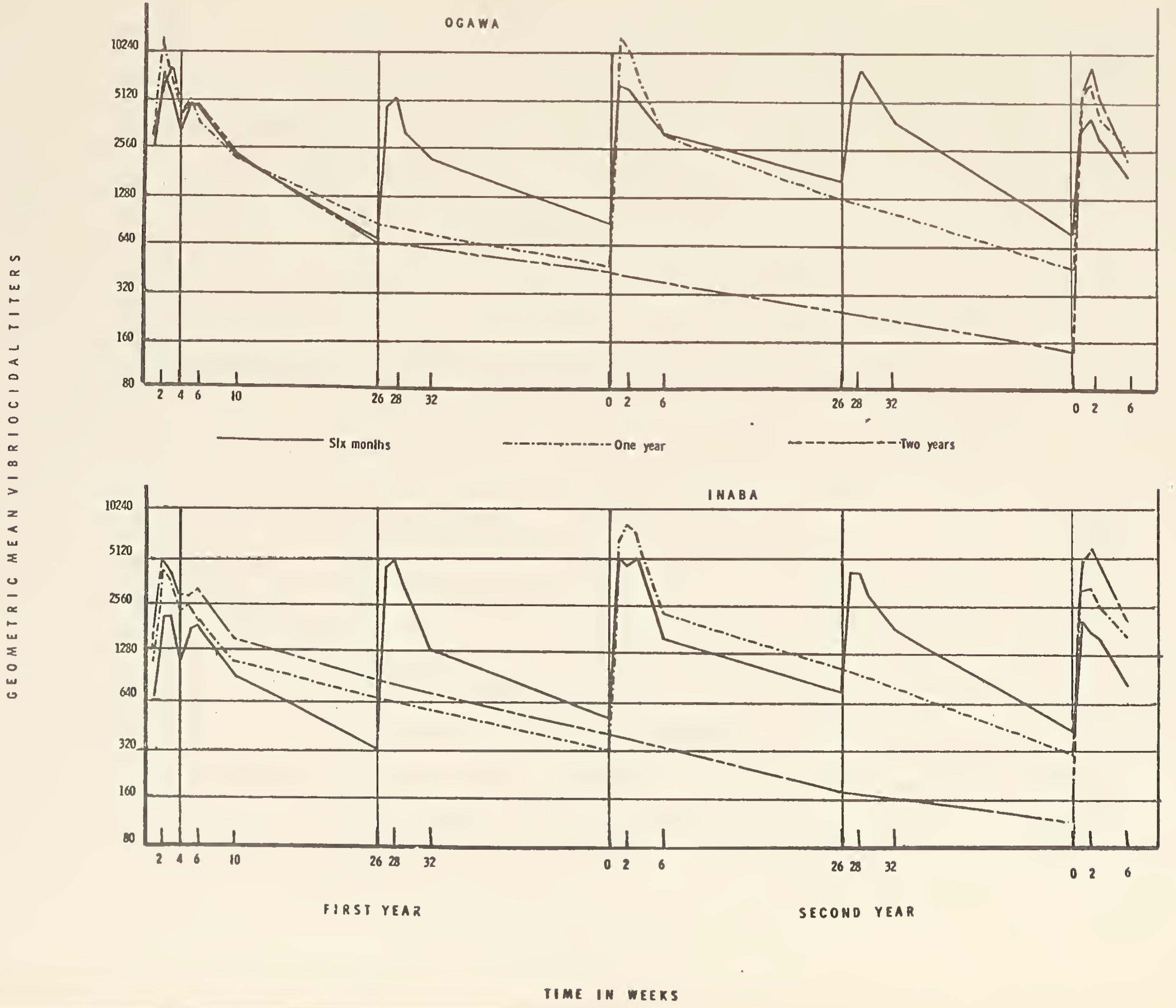


FIGURE 2

GEOMETRIC MEAN VIBRIOCIDAL TITERS OF HUMAN VOLUNTEERS INITIALLY IMMUNIZED WITH TWO DOSES ONE WEEK OR FOUR WEEKS APART AND REIMMUNIZED AT 2 YEARS

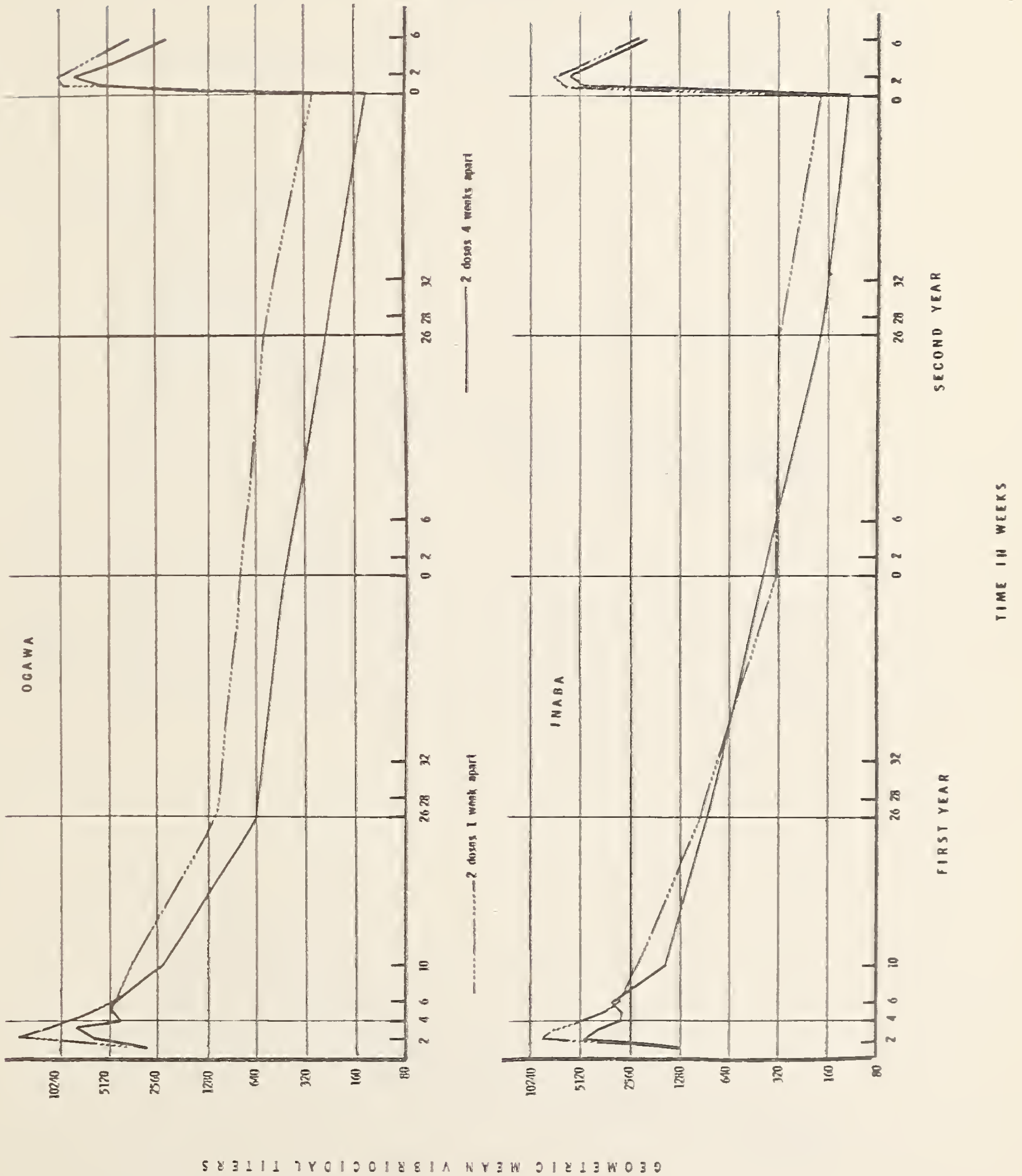
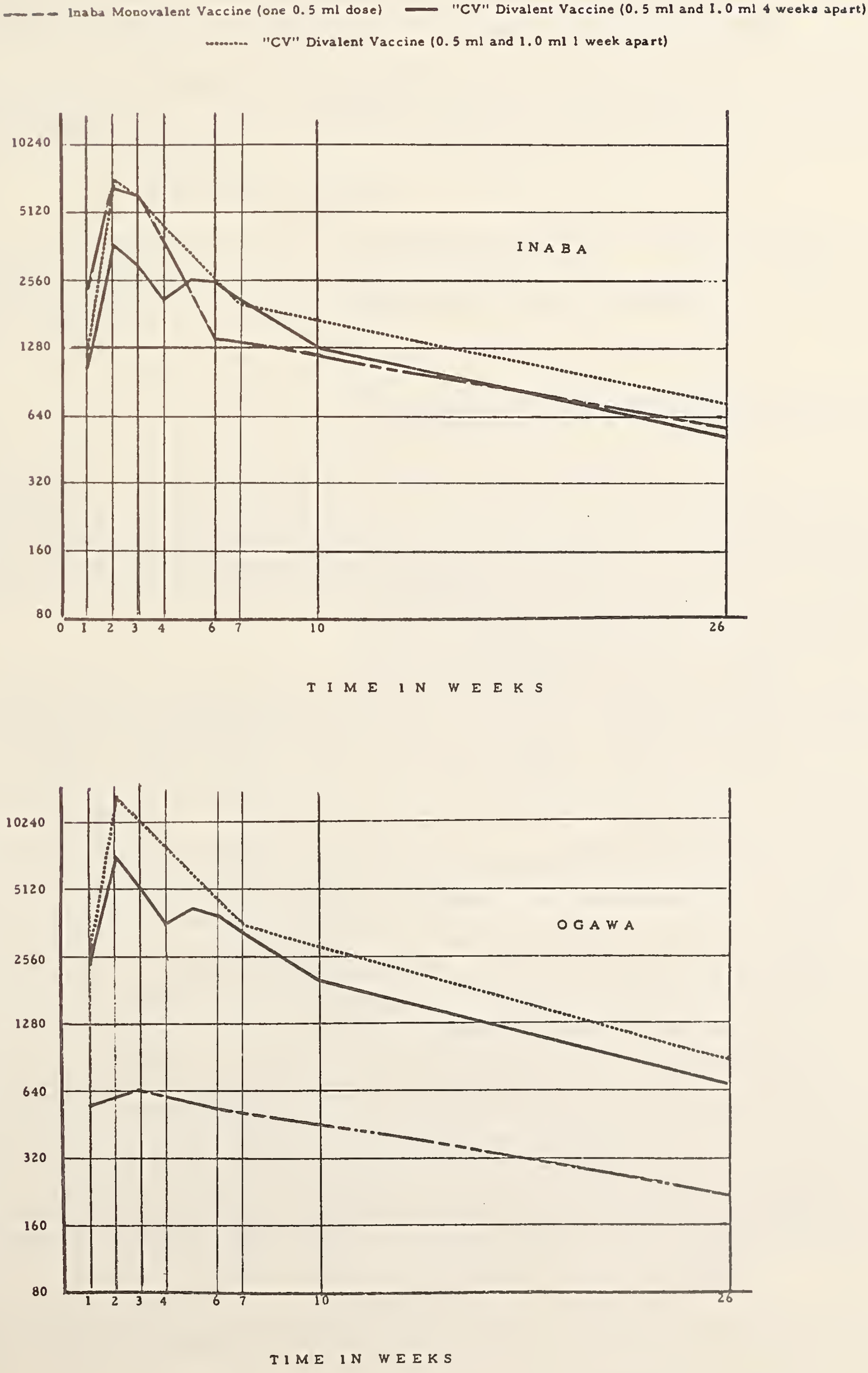


Figure 3

THE VIBRIOCIDAL RESPONSES OF HUMAN VOLUNTEERS TO INITIAL IMMUNIZATION



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13. ABSTRACT

This study of the vibriocidal response to initial immunization and reimmunization with cholera vaccine employed four groups of volunteers, three of which have been immunized with two doses 4 weeks apart and one which received two weekly doses. The three groups have been reimmunized at intervals of 6 months, 1 year and 2 years; and the group immunized at the 1 week interval was reimmunized at 2 years. While further analyses are in progress, the following conclusions are available: 1. Initial immunization with two doses given 1 week apart produces significantly higher peak geometric mean vibriocidal titers than does initial immunization at 4 week intervals. In addition, there appears to be a decrease in the frequency of "poor responders" when the 1 week interval is used. However, these differences are not great and the current recommendation of the Armed Forces permitting an interval of 1-4 weeks between doses for initial immunization appears to be justified. 2. When individuals are reimmunized at 1 year and 2 year intervals, their serological response to immunization is at least as good as that attained by persons who were reimmunized every 6 months. 3. When initial immunization is performed with 2 doses of ordinary vaccine and when reimmunization is carried out with 0.5 ml of similar vaccine, the serological responses that resulted were not materially different from those obtained with volunteers using an Ogawa monovalent vaccine that has been proven to be significantly protective against Inaba and Ogawa cholera epidemics in East Pakistan and the Philippines, respectively. 4. In consideration of the above results, the fact that striking progress has been made in the therapy of cholera, and that cholera vaccination is no longer required for entry into the United States from "a cholera area", it is suggested that the policies of the Armed Forces relating to cholera immunization be reviewed.

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STUDIES ON THE IMMUNIZATION OF MAN AGAINST PLAGUE (U)

FINAL REPORT

by

K. F. Meyer, M.D.

January 1971

(For the period 1 March 1960 to 30 June 1970)

Supported by

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Final Report to the Commission on Immunization of the Armed Forces Epidemiological Board

SUMMARY

The observations here reported disprove the time-honored supposition that standardized tests on mice and guinea pigs can serve as a basis for predicting the protective efficacy of live attenuated plague vaccines in man. The experimental use of highly susceptible nonhuman primates disclosed that the classical E.V. 76 strain is invasive and that it multiplies thereby producing local and systemic reactions as well as tissue injury which may not be merely temporary. Dosages of approximately 1-10th that recommended both in France and the Soviet Union result in measurable clinical reactions in human subjects -- reactions similar to those repeatedly observed but rarely reported (Rotman 1945). Live attenuated plague bacilli probably share with many other live bacterial vaccine properties which may entail risks for some individuals. Some variations in reaction attributable to racial differences have been recorded. For example, Caucasian whites react more severely than Malagasies. Of the live plague vaccines tested, the original E.V. 76 Paris strain is probably the least harmful. It does stimulate hemagglutinins in man (but no significant protective antibodies) following a single inoculation of 52 million viable cells. In this dosage, however, it may act as a booster in human subjects previously inoculated with killed vaccine. Thus, as with killed vaccine, at least 2 (preferably several) inoculations of as yet unknown dosage

may be required to ensure adequate protection. Clinical pilot trials on man with relatively small doses of live vaccines administered intracutaneously by puncture failed to stimulate protective antibodies, even in previously vaccinated individuals. Until standardized studies such as those detailed in this report have been repeated by other investigators, the claim that the cutaneous method of vaccination produces protective immunity in man must be accepted only with reservation. Furthermore, in the clinical trials described, local reactions, even with small doses of viable E.V., were definitely objectionable and persistent -- in 1 individual leading to abscess formation. Previous vaccination apparently failed to diminish the inflammatory reaction in man. The theory that the immunity mechanism activated by killed vaccines could be enhanced by live vaccines received no support from the clinical trials thus far reported. Numerous contentions that guinea pigs or mice acquire protection against plague infection by oral (Korobkova 1970) or conjunctival administration, or by inhalation of aerosol sprays or powders consisting of highly invasive E.V. isolates (E.V. 51f), have been confirmed in a few experiments on nonhuman primates. According to clinical findings reported from the Soviet Union (Pollitzer 1966) aerosol methods of vaccination induced essentially no clinically manifest reactions. The ability of these aerosol vaccination methods to stimulate protective antibodies, however, has not as yet been assessed in man. Furthermore, published data relative to the dosage of 150-200 million organisms necessary to confer the desired protection require confirmation. Until the safety and "innocuousness" of the live P. pestis E.V. has been conclusively proven, clinical trials should be suspended.

The evidence collected through experiments on animals, clinical trials on human subjects and the recent experiences in Vietnam serve to remove the stigma attached to killed plague vaccines designating them as nonprotective against plague. The USP vaccine developed under the auspices of the Commission on Immunization of the Armed Forces Epidemiological Board and standardized according to a recognized assay method in the dosage of 1 ml of 2 billion formalin-killed aqueous suspension highly virulent P. pestis administered subcutaneously or by jet injection, followed by several intramuscular booster inoculations in smaller doses of 0.2-0.5 ml (400 to 1 billion organisms), is a safe vaccine relatively free from local and systemic side effects. Its protective efficacy is readily measured by the passive hemagglutination test. Individual human subjects who acquire hypersensitivity need no booster inoculations; their antibody levels are generally of a high order.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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Introduction: Since plague is essentially a disease of rodents, and since the course of the infection in man is similar to that naturally occurring in rodents, it would seem that laboratory tests to determine the value of prophylactic inoculations would be much easier to perform adequately than actual trials in the field. In fact, such tests are generally considered more instructive in spite of being based on animal experiments, not actual clinical tests on man. In truth, however, so many factors are involved in executing such experiments that to evaluate the results is as difficult as to judge the statistical evidence. In general, through experiments on animals, it has been established that an immunity varying in the degree of completeness can be conferred by inoculation with: (1) suitably killed broth cultures, (2) whole agar-grown suspensions of plague organisms (antigens),(3) certain fractions of such bacilli, or (4) suspensions of living plague strains of attenuated virulence. The latter vaccines in high concentrations are apt to cause unpleasant side effects. Available data are telling, but in evaluating them, one must consider the fact that the animal experiments performed to prove the efficacy of a particular antigen or vaccine have not always been planned to yield complete information. The variables concerned here are (1) the antigen or vaccine being tested, (2) the thoroughness with which the immunization is carried out, and (3) the adequacy of the test dose with which the immunized animals are thereafter infected. These factors in turn are influenced by the choice of experimental animals. The claim that the *kind of test animal* rather than the *kind of immunogenic preparation* is decisive in the results of immunity experiments requires careful appraisal.

I. *Selection of Test Animal Species*:

Through years of tedious experimentation it has been proven that:

(A). Laboratory-bred *white mice* of an inbred stock (old Haffkine, Swiss-Webster) are considered the experimental animal of choice for plague work. They are used to measure quantitatively the virulence or to isolate *P. pestis* from plague infections (Sokhey 1939; Brygoo 1969). Certain strains can be infected with less than 5 plague bacilli. They may be readily immunized with relatively small doses of whole antigenic plague prophylactics or chemical fractions of *P. pestis*. The protection afforded mice by live plague vaccine depends on the number of viable *P. pestis*, or in the case of killed vaccines, on the antigenic mass (mg of N/ml), preferably administered in a two-step inoculation schedule. Quantitative assays of killed plague vaccine lots are made in the so-called Mouse Protection Test (MPT) originally developed by Sokhey and Maurice (1934 and 1935). It has been accepted in a modified form by the Division of Biological Standards of the National Institutes of Health. The ratio of the test to a control reference vaccine is determined according to a standardized procedure outlined in Appendix B. Highly susceptible mouse strains such as NAMRU and the multimammate

(*Praomys natalensis*) have proven useful for studying the virulence of live plague vaccines in the pathology of infection.

(B). Guinea pigs, extensively used in the past to appraise the protective properties of live plague vaccines, are now considered genetically unsuitable because they resist the toxins and fail to develop protection against plague following inoculation with killed vaccines or antigenic fractions. In fact the impossibility of immunizing guinea pigs with killed plague bacilli in saline suspensions or broth cultures served to activate research with living plague strains of relatively low virulence. Now it is known that killed vaccines or soluble antigens (Fraction I), particulated or coated with synergists (aluminum hydroxide) or incorporated in adjuvants, are as active as living attenuated *P. pestis* (Spivak *et al*. 1958). Basic immunization with relatively large doses of killed plague vaccine, or repeated booster inoculations in small doses, may confer a protection equal to that of live vaccines. Experiments to establish comparative potency tests of killed vaccine lots have yielded promising results (Eisler *et al*., in press).

(C). Wild rats (*R. norvegicus*, *R. rattus*, or *R. diardi*) are quite susceptible to toxins and are therefore only suitable for tests on broad lines, not for finer standardization. Various strains of some laboratory-bred albino rats respond irregularly to infection, particularly to titrations of *P. pestis* highly virulent for mice. Hence the protective efficacy of plague vaccines are now measured in mice rather than rats.

(D). Although rabbits are unsuitable for plague immunization studies because of individual variability of response to the infecting dose administered by the subcutaneous or cutaneous route, they readily produce *in vitro* and *in vivo* active antibodies of the highest order and are therefore used in the manufacture of protective antisera or specific antibodies required in the identification of *P. pestis* antigens (FI, V and W).

(E). The selection of two species of *nonhuman primates* highly susceptible to plague infection has greatly aided the study and evaluation of plague vaccines. The inherent individual resistance of *Macaca mulatta* (rhesus), *M. radiatus*, *M. irus* and *Cynomolgus philippinesis* made it impossible to achieve reproducible clear-cut serologic responses to antigen inoculations and to challenge injections (Strong 1907; Taylor 1933; Chen *et al*. 1964). For example, in 1- to 2.5-year-old *Macaca mulatta* infected by the subcutaneous or intracutaneous route, inoculations of 5 million to 194 million *P. pestis* (mouse LD$_{50}$ = 10-20) induced bacteremia or acute or protracted bubonic plague, with *20* to *40* percent surviving. Nevertheless, Otten (1933, p. 148) stated "the problem concerning the protective value of plague vaccines in man is not solved. To approach a solution of the question, human susceptibility to plague infection and response to immunization should be known and on this point reliable information a priori is not available. The monkeys seem to be the most suitable animal to bring this problem nearer solution."

(1) The Hanuman Langur, Presbytis entellus, as a Model of P. pestis Infection.

At the turn of the century, it was recognized (Wyssokowicz and Zabolotny 1897; Gaffky et al. 1899) that the Indian black-faced, long-tailed species Hanuman langur (Semnipithecus or Presbytis entellus) was "very susceptible" to plague. Importation from India and conditioning of this particular species in the U.S.A. were delayed until 1963, when the Government of India, through negotiations conducted by Dr. Henry M. Gelfand, Agency for International Development, and the National Institute of Communicable Diseases in New Delhi, granted export from restricted areas in Mysore. Dr. Leon Schmidt of the National Center for Primate Biology at Davis, California, assumed responsibility for the conditioning of small shipments (12 to 20) of langurs. Serious losses of these valuable experimental animals were sustained. Of 100 Hanuman langurs, only 52 were salvaged. Nine of the 48 colonization failures occurred within 48 hours after arrival and 15 within the first 10 days in this country. Twenty-four animals were cachectic, with diarrhea and overt upper respiratory tract infections. Of the remaining 24 fatalities, 21 must be considered a consequence of the sequelae of slow starvation, since they found neither the food nor care provided acceptable. Eleven succumbed to enteric infection with Shigella flexneri, and two died of pneumococcal meningitis, Type 33. The decision to select the summer months for shipments and the accidental discovery that the feeding of fresh green alfalfa, fresh green beans, and broccoli supplemented by Primate Center ration and Purina monkey chow resulted in considerable improvement of health. Housing in pairs in single cages in a special room with a temperature of 15°C "miraculously" imoroved colonization. Usually conditioning combined with chemotherapy required from 4 to 6 months. The problems were apparently solved when the absence of trapping facilities in the reserve provinces and other irregularities attributable to the importing dealer prevented further importation of the animals after 1967. As a substitute, a shipment of silver leaf langurs (Presbytis cristatus) was received. The experience with this shipment was devastating: of 105 langurs, only 35 were ultimately available for experimental use. All were in poor physical condition upon arrival from Malaysia; 19 died within 48 hours of arrival and 22 within the next 8 days. All deaths were essentially the result of starvation or overwhelming pneumonia caused by Klebsiella pneumoniae, Types 1 and 11. Most of the fatalities between the 11th and 60th days were also attributable to pneumonia, associated with emphysema, pericarditis, and, in a surprisingly high incidence, peritonitis --

invariably caused by Klebsiella sp. Penicillin and streptomycin in large doses administered for 2 to 8 weeks proved ineffective. Animals surviving 60 days in the colony were without exception in good physical condition and required large amounts of leafy food, particularly alfalfa, in their diet.

The number of well conditioned Hanuman langurs available limited two susceptibility tests to 26 animals, which were each infected subcutaneously with one of 5 dilutions of 102 to 125,000 viable P. pestis 195/P (mouse LD_{50} = 2). The course of the disease and time of death were not related to the dosage of the inoculum. Two langurs receiving 102 and 1020 organisms, respectively, and 2 out of 4 receiving 125,000 bacilli, died within 8 to 12 days. The two receiving 10,200 P. pestis both succumbed within 9 days. It is obvious that langurs, like macaques, exhibit both high and low resistance to P. pestis. The relatively small dose of 100 to 1,000 P. pestis may cause death, or alternately, disease with survival and immunity. A dosage of more than 100,000 organisms caused disease, septicemia, and 50 percent mortality. Thus despite variable individual susceptibility, it may be concluded that Indian langurs are more susceptible to subcutaneous plague infection than rhesus or Cynomolgus monkeys. The clinical course and autopsy findings resembled those of plague in man. Septicemia was an early symptom, primary buboes were of moderate size except in protracted infections, but the tendency to hemorrhage was more marked than in other species of nonhuman primates. Strong humoral immunity followed recovery from infection and was reflected in significant levels of passive hemagglutinins, complement-fixing antibodies, and low-level (4 to 8) mouse protection indices.

In a second experiment, the infective units varied from 82 to 81,000 virulent plague bacilli. Four animals inoculated with 81,000, 2 with 810,000 and 2 with 810 virulent organisms, died on the 4th to 8th day with the typical lesions of plague. Two inoculated with 81 P. pestis remained well and revealed no serum antibodies. They succumbed to plague after challenge with 64,000 virulent P. pestis. Aside from procurement difficulties, the cost of accurately determining the LD50 of P. pestis in Hanuman langurs would be prohibitive. Even tentative LD_{50}'s could not be calculated. An average infective dose of 300,000 P. pestis, which on the 4th to the 6th day caused fatal plague in Hanuman langurs not previously inoculated with plague antigens, was chosen for challenge. The first pilot plague immunization experiment with an adequate number of langurs was performed with the combined plague-cholera-typhus or "exotic package" vaccine. A single dose (1 ml), followed by a booster inoculation (0.2 ml) on the 3rd month, conferred protection against an 81,000-organism subcutaneous infection in 2/3, or against an 810,000-organism subcutaneous infection in 1/4. The serum antibody response was modest; no complement-fixing antibodies were evident, but hemagglutinins varying in titer from 1:8 to 1:32, as well as a drop in the average MPI from 13 to 8 were observed. Two months after recovery from challenge infection, the animals had CF antibody titers from 1:2 to 1:32, hemagglutinins of 1:8 to 1:512, and an average MPI of 4 (Report 16). A second pilot experiment on Hanuman langurs, with two experimental lots of killed plague U.S.P. vaccine grown on E. medium, demonstrated that one preparation (E-34819), administered in two doses, failed to confer protection. The second batch conferred a solid immunity upon 4 out of 4 animals. In an additional experiment, 9 Hanuman langurs were each inoculated with U.S. plague vaccine in two doses three months apart and infected with 131,000 virulent P. pestis. Six (66 percent) of the Hanuman species survived; they exhibited either well defined local reactions or none. The course of the disease in three of the vaccinated animals in comparison with the controls was protracted and complicated by metastatic pneumonia. Deaths occurred on the 16th, 21st, and 35th days. This protection experiment on a small group of langurs again indicated that the species is definitely both more susceptible and more uniform in reaction to plague than is Macaca mulatta. The capacity for immunization of individual members, however, is heterogeneous. As a model for evaluating the immunity mechanism against plague, the Hanuman langur may confidently be put in first place. Resistance is indirectly reflected by the Mouse Protection Indices; none of the vaccinated individuals with a MPI above 10 survived. The appearance of CF antibodies (1:16 to 1:64) after challenge infection is noteworthy (Report 18, p. 11).

Important information concerning the protective and immunogenic potency of live attenuated plague vaccine was acquired from Hanuman langurs. Groups of 3 to 4 well conditioned animals were inoculated intradermally with dilutions of reconstituted freeze-dried vaccine containing 17,800, 178,000 or 1,780,000 viable P. pestis E.V. (WR-Saigon). Two of the nonhuman primates receiving the large dose acquired erythematous areas (5 x 10 mm) with 2 x 2 mm indurated centers which persisted for 4 to 6 days. Aside from slight fluctuations in weight, no illness or fever was noted. Antibody response, already vigorous on the 8th day, and CF and HA titers by the 66th day after inoculation as well as MPI indices all reflected good immunity. In view of the observation in guinea pigs that as few as 400 viable organisms of the P. pestis E.V. (WR-S) isolate protected 9/10 against a 100,000 LD_{50} (1,250,000 P. pestis) subcutaneous virulent infection, the outcome of the experiment on langurs was surprising. A single inoculation of the live vaccine, regardless of dose, protected only 5/11, or 45 percent of the animals. The groups of langurs vaccinated are too small for the calculation of meaningful correlations between the vaccine dose and the state of resistance induced. In the search for a satisfactory explanation, it was recalled that killed vaccines in a single dose generally confer a low percentage of protection, which, however, can be enhanced by booster inoculations. Serological surveys in Madagascar (Payne *et al*. 1956) on human subjects demonstrated that basic immunizations with live attenuated E.V. strains elicit antibodies in relatively few persons, but that subsequent re-inoculations of the vaccine progressively enhance the production and maintenance of antibodies and correspondingly the resistance to infection (Report 17, p. 25). Two experiments, one with *Macaca mulatta* (Report 17, pp. 26-28) and one with Hanuman langurs, indicate that the principle of *two inoculations (the so-called booster schedule) applies equally to live E.V. as well as to dead vaccines*. Six langurs were each subcutaneously inoculated with 53,000,000 viable *P. pestis* E.V. Saigon in a total of 270 million organisms, while one *Presbytis* received 5,300,000 bacilli. On the 50th day after this basic vaccination, the second (booster) inoculation of 4,450,000 viable organisms in a total of 30 million given to three animals moderately influenced the antibody response. The difference in resistance to a subcutaneous infection with 420,000 virulent *P. pestis* 195/P between animals receiving single vaccinations and revaccinated animals was not striking. All controls succumbed on the 4th or 5th day, with lesions and bacteriologic findings of acute septicemic plague. Only one non-boostered langur, with septicemia on the 5th day, succumbed to plague on the 9th day. The resistance conferred by the vaccine upon 6 of 7 langurs was not absolute. All animals, whether boostered or not, exhibited local inflammatory lesions varying in diameter from 3 to 7 cm at the site of infection. The centers of the swellings became necrotic and discharged pus containing virulent *P. pestis*. Lymphadenopathy accompanied the local lesions; in one langur not boostered, the abscess in the right axillary lymph node had perforated by the 16th day. Although the immunity-enhancing effect of a small booster inoculation following a relatively large basic vaccination with the E.V. Saigon isolate was not conclusively proven, another factor deserves consideration here -- the same vaccine in identical dosages subcutaneously administered to human subjects produced erythemas which persisted for 72 hours; in one individual, a painful induration and a slight rise in the white blood cell count 24-48 hours after inoculation. The antibody response even after booster was zero except in one individual previously vaccinated. A second booster inoculation with killed U.S.P. vaccine stimulated the appearance of hemagglutinins but had no effect on the Mouse Protection Index. The live vaccine sensitized the immunity mechanism only slightly. Thus the prevailing basic concept - that live plague vaccines with an excellent presumptive efficacy in mice, guinea pigs and a highly susceptible nonhuman primate, the langur, may be used to immunize man - must be accepted only with definite reservations (Report 18, p. 44).

It was assumed that the disappointing immunogenic response of human subjects to intracutaneous or subcutaneous vaccination was attributable to low-dose inoculations. To test this concept, 25 men were vaccinated with the standard dose (approximately 500 million organisms) as originally recommended by Girard. Simultaneously a series of Hanuman langurs were similarly inoculated with vaccine dilutions containing 420,000,000 and 42,000,000, respectively, live *P. pestis* E.V. Saigon in a total suspension of 2 billion and 200 million plague bacilli, respectively. Small nodules or edematous indurations extended over the femora for 72 to 96 hours, with no impairment of function or general health; enlargement of the lymph nodes could not be ascertained. Antibody response was excellent, with individual HA titers reaching as high as 1:32,768; CF titers of 1:4 to 1:64; and, except in the case of two animals, Mouse Protection Indices all below 5 on the 28th day after vaccination. Since experience had demonstrated that langurs with high-level antibodies survived severe challenge, it was decided to use this experiment to determine the duration of immunity (Report 18, p. 46). Over a period of 13 months, antibodies were measured on the 93rd, 126th, 177th (approximately 6 months), and 406th days (approximately 13 months) after vaccination. The average MPI rose from 2.5 on the 28th day to 8.4 on the 406th day; in *some* individual langurs, it *returned to normal levels*. Hemagglutinins persisted but likewise declined from an average of 1:4554 to 1:1335 in the 13th month. In groups of 8 langurs, each receiving a booster inoculation on the 406th day, the elevation of antibodies within 11 days was striking, but by the 85th day after re-inoculation, a decline in titer was again recorded. The antibody recall response to approximately the same vaccine dosage (45 million organisms) as that of the original vaccination (42 million organisms) was *more pronounced* than in the group vaccinated with the larger dose of vaccine (420 million organisms). It is suspected that in an as yet unexplained manner the large vaccine dose so clinically innocuous injured the antibody producing mechanism. The resistance of the vaccinated langurs to a severe subcutaneous challenge infection (307,000 *P. pestis* 195/P) given during the 16th month after original vaccination was significant and unexpected in view of previous reports: *10/15* (66 percent) survived the infective dose which was fatal to 3 controls on the 3rd to 6th day. Seven of the survivors exhibited solid immunity. The five fatal infections occurred between the 10th and 12th days and were complicated either by metastatic pneumonia or by pregnancy -- a danger frequently observed in humans in the course of every plague epidemic, ancient or modern. The groups of langurs available for this experiment were too small to gauge adequately the efficacy of the booster inoculations. In Group I, vaccinated with single doses of 420 million organisms, 1/3 survived, while in the boostered, 3/4 proved immune. In Group II, vaccinated with 42 million organisms, either with or without a booster, proved in part to be protected. The four nonboostered langurs were afforded some degree of protection; their disease (bubonic-septicemic plague) was protracted and the deaths complicated by metastatic pneumonic consolidations of varying extent and intensity. No systematic temperature measurements were made, but from previous experiments it is known that the institution of chemotherapy when fever was recorded would have cured the slowly progressing infections which are usually rapidly fatal to nonvaccinated animals.

Previous studies (Report 18, p. 42) had demonstrated that the same vaccine in a dosage of 53,000,000 organisms on the *100th* day after inoculation protected 6/7 langurs against fatal infection without local plague reactions. The present experiment revealed that the *absolute protection conferred upon subhuman primates by a live partially deteriorated vaccine may persist in individual animals for at least 16 months*. This resistance is indirectly reflected by protective antibody levels. By the end of the 3rd month after basic vaccination, the MPI of individual animals began to fluctuate, and by the 12th month it reached relatively high levels. Revaccination restored the original level. However, experiments conducted on small groups of langurs fail to furnish conclusive information concerning the booster effect afforded by revaccination with live vaccine -- an effect which is invariably demonstrated with killed vaccines. Furthermore, it should be emphasized that these studies on the duration of immunity in langurs were conducted with a vaccine which proved to be incompletely immunogenic in man (Report 19, p. 10). The outcome of experiments measuring the duration of immunity conferred upon langurs by *P. pestis* E.V. Saigon warranted measuring the degree of protection against aerosol infection afforded by subcutaneous and booster vaccination. Four langurs (weighing 6-8 kg each) were inoculated subcutaneously with 45-55 million viable *P. pestis* E.V. 76 Saigon, boostered with 60.5 million viable organisms of the same vaccine 18 weeks after primary inoculation, and then exposed to aerosol infection (*P. pestis* KIM-10, inhaled dose 8.1 x 10^5) at Fort Detrick. Only one animal survived the aerosol exposure.

When the vaccinated and nonvaccinated animals were compared, marked differences in survival times after exposure to very severe aerosol infection were observed. Langurs without *P. pestis* serum antibodies died within 3 days. Three of the four vaccinated langurs succumbed in 6 to 10 days, with the typical lesions of pneumonic plague characterized by microscopically visible alveolar flooding with proteinaceous fluid, some hemorrhage, mild to severe polymorphonuclear cell infiltration, luxuriant bacterial overgrowth, and necrotizing bronchiolitis -- in some animals accompanied by bronchial lymph node edema or hemorrhages, with lymph node necrosis. The langur which survived the exposure, sacrificed on the 21st day, exhibited the residual signs of focal pneumonia. Some form of immunity had prevented the progressive growth of the infective plague bacilli. Experience had indicated that antibody titers (MPI oo to 1) of the magnitude recorded following inoculation of langurs with live plague vaccine invariably conferred a *solid* protection against *subcutaneous* plague infection. The single experiment revealed that this correlation may not apply to infections contracted via the respiratory tract. However, the workers who performed the challenge test stated that the infective doses were much higher than intended. Nevertheless, the vaccine inoculation afforded incomplete protection to 3/4 animals, with some specific immunity, as indicated by disease periods slightly longer than in the nonvaccinated langurs. In this connection, it is important to recall that the E.V. Saigon isolate proved to be partially deficient in vaccination experiments and clinical trials performed on nonhuman primates and on man by either the cutaneous or subcutaneous route. Doubtless of greater significance were the pilot experiments demonstrating that macaques passively immunized with highly concentrated anti-*P. pestis* rabbit serum globulins are rarely protected against intratracheal infection with virulent *P. pestis* (Ehrenkranz and Meyer 1955). On the other hand, guinea pigs and mice immunized by the respiratory or by the ocular route are afforded solid protection against aerosol infection, notwithstanding the fact that peripheral serum antibodies may be either absent or of a low order (Soviet studies). Thus the current experiment on langurs supports the view that antibodies alone may be inadequate to prevent the multiplication of *P. pestis* in the respiratory tract. In addition, this experiment lends conviction to the slowly growing realization that attempts to protect against respiratory infections by cutaneous and subcutaneous vaccination are immunologically unsound.

The accidental discovery that a living *P. pestis* E.V. 51, which, in its 6th passage through guinea pigs pretreated with nontoxic doses of iron salts, was isolated from a spleen with typical granuloma, proved fatal for African vervets (*Cercopithecus aethiops*) necessitated obtaining virulence titrations on Hanuman langurs. This isolate, designated *P. pestis* 51f, was nonlethal to guinea pigs in the dosage of 1,000,000 organisms, but caused the death of 1/4 Ethiopian *Cercopithecus* (weighing 4.7 to 6.6 kg each) in the subcutaneous dosage of 16 million bacilli. Vervets from Kenya proved more susceptible. The following mortalities were recorded: 218 million organisms, 3/4; 2.18 million, 3/4; 218,000, 2/4; and 2,180, 2/4. The relatively small dose of 218 viable organisms effectively immunized 4/4 vervets (Report 18, pp. 57-66). An independent recheck of these findings by workers at the South African Institute for Medical Research disclosed that according to their records, 13/50 (23 percent) *Cercopithecus aethiops pygerythrus* had succumbed to subcutaneous vaccination. In the group of 10 animals inoculated with either 100 million or 100,000 organisms, the mortality rate was 40 percent. A study of the effects of E.V. 51f on four Hanuman langurs was undertaken to determine and measure local and systemic reactions. In retrospect, it appears that valuable animals were needlessly sacrificed, since the outcome was predictable from preceding experiments on *Cercopitheci*. The vaccine dose (130 million viable organisms in a total of 707 million) represented the approximate number of viable *P. pestis* E.V. 51f to be tried on human subjects. It was soon discovered that the vaccine caused severe, painful, local inflammatory reactions which persisted for more than 2 weeks; in fact, nodules could be palpated as late as the 25th day after inoculation. In contrast, local lymphadenopathy was not as marked when vaccination was accomplished by the cutaneous route. By the 3rd day, the animals were lethargic; they ate vegetables but no biscuits. All displayed fever, and 2/4 langurs which succumbed on the 6th to 7th day yielded positive blood cultures. Gross anatomic lesions at autopsy resembled those of acute bubonic-sepitcemic plague, with significant exceptions: little or no hemorrhage at the site of inoculation, extending to the regional lymph nodes, which were enlarged, grayish, or very slightly congested but not embedded in edema or hemorrhage. Thoracic, pericardial and abdominal cavities were free of fluid. Enlargement of the spleen, parenchymatous changes in the liver and kidneys, some hemorrhage in the adrenals, and congestion of the lungs with edema and dilatation of the ventricles and auricles of the heart, were alterations indistinguishable from those of virulent plague. The animals which survived vaccination were solidly protected against a severe subcutaneous virulent infection (307,000 organisms) with *P. pestis* 195/P.

It is claimed by Soviet immunologists that the cutaneous technique of vaccination with live E.V. plague vaccine reduces the unpleasant side effects. The original purpose of the next experiment, using the intradermal puncture technique, was (1) to gauge the intensity of the local reactions anticipated from previous experiments on langurs and vervets, and (2) to compare approximate doses required for specific protection. Well-conditioned langurs weighing from 5 to 8 kg each, and *Macaca mulatta* (3-5 kg), were shaved on the chest over the xiphoid bone and the skin was cleansed with alcohol. New sterile bifurcated needles were dipped into the reconstituted diluted vaccine contained in small Petri dishes to a level where only the space between the two forks became filled with fluid. The needle was gently pushed into the skin and rapidly withdrawn without extracting blood. Carefully prepared cultures of the vaccine suspension retained in the needles indicated that approximately 842,000, 84,000 or 8,000 viable E.V. 51f organisms, respectively, were delivered to the puncture wound. Parallel tests on guinea pigs revealed that in this technique with the needle charged with 8,000 organisms, 9/9 guinea pigs were protected. Although a separate needle was used for each animal, it was subsequently discovered that the needle-points were of variable sharpness. Thus a single puncture might not deliver the desired vaccine dose. As usual, plague studies on langurs resulted in unexpected findings: (1) Local reactions in properly vaccinated animals were manifest as pinhead-sized, pink-to-deep-red nodules apparent by the 24th hour and subsiding to faintly visible needlemarks by the 5th day. At autopsy, neither the skin nor subcutaneous tissues revealed inflammatory reactions at the site of puncture. (2) Within 5 days, the axillary lymph nodes (either the right or left) were palpable and eventually attained the size of large peas or even small peanuts, persisting for more than 20 days in the only survivors. (3) Beginning on the 2nd day in one langur vaccinated with 800,000 organisms, and in three others on the 5th to 9th days, the body temperature rose to 40^{o}C or above. (4) After the 5th day, successfully vaccinated monkeys were clinically ill and decidedly less active, consumed less food, and finally exhibited complete anorexia; all had diarrhea. (5) Daily quantitative blood cultures yielded blood-plate surface colonies of typical E.V. 51f varying from 24 to 200/ml. (6) Biopsy aspirates of the enlarged axillary lymph nodes on the 12th day invariably produced heavy growth on blood plates, but only one revealed typical plague bacilli in Giemsa-stained smears obtained for studying the types of monocytes in the nodes. (7) As early as the 8th day after vaccination, hemagglutinins appeared in titers of 1:64 to 1:2048. (8) Between the 12th to 17th day, 4 of 5 animals successfully punctured with approximately 80,000 or 800,000 E.V. 51f succumbed, with unique gross anatomical lesions of acute plague complicated by enteritis of varying intensity, but with no evidence of the pleural and pericardial exudate common to virulent infections. Lesions in the enlarged axillary lymph nodes draining the vaccination areas were particularly striking. They were firm, grayish, granulomatous, and even necrotic, without hemorrhages except in biopsied animals, and with no encircling edema. The spleen was always enlarged and pulpy; in an animal punctured with 800,000 organisms, it was exceptionally large, accompanied by the necrosis-studded liver commonly seen in African green monkeys subjected to attenuated plague inoculation. With one exception, metastatic pneumonic areas of consolidation varying in extent were present in the poorly collapsed lungs. Right cardiac dilatation due to partially coagulated blood was observed in every animal. This manifestation of toxemia was complicated by hemorrhagic gastroenteritis. In one langur, bleeding from the nasal passages and upper respiratory tract involved the right anterior lobe of the lung. Parenchymatous changes in the kidney -- nephritis, urine with high protein content -- and adrenal hemorrhage were similar to those seen in virulent infections. (9) Bacteriologic examinations employing 4 different culture media (including Gentian violet, McConkey agar plates) revealed P. pestis 51f in all organs and tissues, generally mixed with E. coli and Klebsiella. Even the heart blood, liver, spleen and axillary lymph nodes, carefully removed from the carcass of one langur sacrificed while moribund, yielded isolated colonies of E. coli and Klebsiella, as well as a heavy growth of P. pestis. (10) Of the 2/6 langurs surviving vaccination, one had no local reactions or bacteremia, developed no antibodies, and subsequently succumbed to virulent infection; the other, after recovering from an 800,000-dose puncture vaccination, proved solidly immune when challenged 167 days (24 weeks) after inoculation with 343,000 virulent P. pestis 195/P.

Comparing the response of Macaca mulatta to E.V. 51f intradermally administered with a single bifurcated needle puncture to the reaction observed in langurs, the former species low susceptibility to plague was again strikingly apparent. No local cutaneous reactions, enlargement of regional lymph nodes, or systemic effects could be detected. The animals stayed well, and repeated blood cultures remained sterile. Nevertheless, the antibody response was vigorous in 3 macaques with MPI of 5 or below, and they proved to be solidly protected against subcutaneous infection. The single puncture apparently failed to produce infection.

On the basis of this single pilot experiment with macaques, the E.V. 51f isolate would appear to be a safe, highly efficient vaccine, producing no local or systemic reactions. A contradictory interpretation, however, emerged from analysis of the clinical test employing langurs. Although local reactions produced by the intradermal puncture technique were slight, it was disconcerting to note that in this highly susceptible species, the E.V. 51f proved highly invasive, causing slowly developing generalized infection, reflected by positive blood cultures beginning on the 5th day after inoculation as well as rapid enlargement of regional lymph nodes. Progressive toxemia and persistent bacteremia attest to the "residual virulence." The "vaccination disease" led to death on the 12th, 13th and 17th days after puncture vaccination in a sequence of events completely compatible with the usual course of protracted infections with virulent P. pestis (Report 19, pp. 18-25).

The disturbing results of vaccination experiments using descendent isolates of P. pestis E.V. 76 (Saigon or Madagascar guinea pig passage) on subhuman primates logically required the testing of a transplant of the original culture maintained by Dr. G. Girard at the Institut Pasteur. This transplant was obtained in December 1967. The PD_{50} of the E.V. 76 Paris was 800 when it was used in a vaccination experiment involving 4 Hanuman langurs, 6 African vervets, and 4 macaques, each intradermally inoculated with a suspension of 26,800,000 viable organisms in 0.1 ml. Regardless of the weight (8-15 kg) and sex of individual monkeys, local reactions in the form of erythematous indurated areas (2 x 2 cm) apparent by the 48th hour after inoculation, had begun to fade by the 5th day, but indurations were still marked on the 8th day, becoming palpable as nodules on the 16th day and hairless spots (0.9 x 0.9 cm) by the 27th day. By the 48th hour,left inguinal lymph nodes (average size 2.5 x 1.2 cm) were already painful and firm but movable. In one langur, severely ill for 3 days, there was also lymphadenopathy in the right inguinal nodes, which continued to enlarge until the 16th day; in one large langur, these nodes measured 5 x 3 cm each. On the 27th day, the nodes (now 4 x 2 cm) had only moderately receded, but they had become softer in consistency. Reactions were of the same magnitude as those observed in the series of langurs vaccinated with small doses of the more invasive E.V. 51f. Painful local effects were accompanied by systemic reactions in the form of 1-2^0 elevations in body temperature, moderate leukocytosis, anorexia, loss of aggressiveness for at least 5 days, and diarrhea in the smaller animals. The recovery period extended over 8 days. Quantitative blood cultures yielded 2 to 16 colonies in each of the 2 young langurs on the 2nd, 4th and 8th days. On the 5th day, biopsy specimens from the lymph nodes of 3 animals revealed a moderate to abundant growth of the vaccine strain, although careful microscopic examination of the smears, disclosing arrays of monocytes and lymphocytes, proved otherwise unrewarding.

By the 8th day after vaccination, HA antibody titers were already significantly high, and by the 27th day, the appearance of complement-fixing antibodies in titers up to 1:128, together with high HA levels and MPI below 10, conclusively contradicted reports that live attenuated plague vaccines fail to induce peripheral antibodies.

On the 42nd day after vaccination, immunity tests by subcutaneous (xiphoid region) challenge with 343,000 *P. pestis* 195/P revealed absolute protection, exemplified by the absence of local reactions in the skin and regional axillary lymph nodes. All nonvaccinated controls, which died on the 4th day, displayed extensive hemorrhagic necrotizing dermatitis and lymphadenitis.

(2) The Malayan langur, Presbytis cristatus, as a model of P. pestis infection.

A single infection and immunization experiment with the silver leaf Malayan langur (*Presbytis cristatus*) indicates that this species may represent an advantageous test animal for studies with *P. pestis*. This langur is apparently more susceptible than the Indian species. Infection with 81 virulent bacilli proved fatal on the 6th to 8th day. It was not surprising that only 1/4 Malayan langurs inoculated with the killed triple vaccine of established immunogenicity for Hanuman langurs survived a challenge of 81,000 organisms, and 2/3 survived a dosage of 8,100. The latter two were making a good recovery and eating well, when they suddenly succumbed on the 19th and 27th days after challenge, respectively, as a result of apparent endotoxin damage to the liver, kidneys and adrenals, accompanied by profuse growth of *P. pestis* in the lymph nodes, spleen, liver and kidneys but not in the heart blood. The reasons for this sequence of events are not understood but merely suggest that the silver leaf langur may be uniquely promising as a tool for studies with *P. pestis* -- a circumstance which may be exploited by those willing to pay a relatively high cost in dollars and personal attention (Report 16).

(3) The African vervet, Cercopithecus aethiops sp.,as a model of P. pestis infection.

Intermittently shipped imports and tedious, expensive conditioning procedures have limited experimentation to relatively small groups of langurs, thus restricting the scale of studies which may ultimately yield meaningful conclusions. According to published records and inquiries made in South Africa, the plague susceptibility of the common African green or vervet monkey (Cercopithecus aethiops) has never been determined. Since this genus is readily obtainable, a few preliminary tests have been conducted. The susceptibility of the Ethiopian Cercopithecus (weighing 2.4 to 4.9 kg) to subcutaneous plague infection with dosages varying from 200 to 2 million virulent P. pestis 195/P was found to be heterogeneous but, in general, similar to that of langurs in terms of individual resistance, course of the disease, times of death, and autopsy findings. Two vervets each succumbed to infection with 2,000 P. pestis (on the 5th and 9th days), and three others died on the 3rd, 4th and 11th days, respectively, following subcutaneous inoculations of 200,000 organisms. On the other hand, only one out of two became fatally infected with 20,000 or 200,000 organisms. The relatively small dose of 200 organisms caused one death on the 9th day, and by the 29th day the remaining two animals in this group had chronic septicemic plague complicated by right principal lobar, left upper lobar, and ultimately fatal metastatic pneumonia, with pleurisy in the left principal lobe. On the 21st day after infection, the immunologic response was already evident in the form of HA and low MPI, but no CF antibodies. In comparing the serologic reactions, it was noted that they were of a slightly lower order than those in the vervets surviving larger infective dosages. This pilot susceptibility experiment encouraged the investigators to use African vervet species as substitutes for langurs. However, there were surprises ahead.

Cercopithecus aethiops from Kenya was included in experimental trials to evaluate the immunogenic and protective efficacy of living E.V. (WR-S) in langurs and human subjects. At the time of the clinical trial, a vaccine suspension in doses of 53.5 million and 535,000 organisms, identical to that used to inoculate 7 langurs, was administered simultaneously to members of a small shipment of 6 Cercopitheci recently imported from Kenya and conditioned and held at the National Center for Primate Biology. The inoculations were well tolerated, but the antibody response 8, 16, 28, and 50 days after vaccination was negligible. Four subhuman primates of the experimental series received the usual booster inoculation (4.45 million organisms). On the 49th day after the booster, the day before challenge, peripheral blood sera yielded no significant antibodies. The average MPI remained at pre-vaccination level (16). The large infective dose (420,000 organisms) of virulent P. pestis was chosen intentionally, since one of two Cercopitheci from Ethiopia had successfully resisted an infection with 209,000 virulent plague bacilli. The entire group of vaccinated Kenya vervets succumbed to acute septicemic plague either shortly before or following the death of the only available control animal.

The failure to respond to vaccination was surprising for the following reasons: (a) In a comparable experiment, the E.V. (WR-S) vaccine had afforded good protection to langurs (Report 18, pp. 46-49); (b) however, the same live E.V. 76 (WR-S) in large basic and booster vaccine doses inoculated subcutaneously in human subjects proved equally non-immunogenic. HA, repeatedly assessed by the highly sensitive titration technique using formalin-treated sheep red cells coated with Fraction I protein, could not be demonstrated except in one animal vaccinated with dead vaccine 17 years previously, wherein the HA reached a level of 1:16. The mouse protection indices (MPI) before and after inoculation remained essentially the same.

This experiment on a small group of Kenya vervet monkeys fully confirmed the observations from studies on man; moreover it proved conclusively that the live E.V. 76 Saigon in a single dosage of 50 million viable cells, followed by a booster dose of 4.45 million organisms, confers no protection against plague. Thus it was tempting to conclude that certain Cercopithecus aethiops species may represent the best animal model for evaluating the immunogenic and protective efficacy of live, and possibly killed, plague vaccines. Large-scale experiments were cancelled because of the concern which developed following an outbreak of hemorrhagic disease among laboratory personnel who handled tissues or came into contact with blood from Cercopithecus aethiops from Uganda in 1967 (Saenz 1969). In addition, the conditioned vervets available were urgently required for other experiments.

In the course of stabilization and enhancement of the immunogenic efficacy of E.V. 76 strain, a passage isolate -- P. pestis E.V. 51f -- with a guinea pig PD_{50} of 5 viable cells was obtained. Intraperitoneal inoculations of 100 million organisms resulted in the deaths of 2/5 guinea pigs from a combination of inocular invasiveness and toxicity. A vaccination study with E.V. 51f followed by a protection test with virulent P. pestis yielded unexpected results.

Male vervet monkeys imported from Ethiopia were chosen, since pilot experiments had indicated that this genus is probably slightly more susceptible than the langur (Presbytis entellus). Five groups of monkeys were subcutaneously inoculated with saline suspensions of the isolate (grown at 37°C for 48 hours on HIV agar) above the left femoral muscle, thus permitting frequent examinations for local reactions with the smallest possible amount of handling (Report 18). Descending doses of 160 million to 16 million organisms, or 1.6 million to 160,000 and saline were injected. Only the large dose of 160 million viable organisms produced local reactions in the form of erythema and indurations. Most remarkable, however, was the fact that all the subhuman primates of groups receiving from 1.6 to 160 million viable attenuated P. pestis became visibly ill on the 3rd to 4th day with listlessness, glassy eyes, photophobia, anorexia, and weight loss for 8 days or longer. Two (one inoculated with 160 million and one with 1.6 million organisms) died on the 5th and 6th day, respectively, with generalized culturally proven septicemia and splenic tumor (42 gm, five times the normal weight); whereas at autopsy, a third animal (inoculated with 1.6 million organisms), sacrificed while moribund on the 18th day, revealed classical granulomas in the spleen and liver -- lesions originally considered by Girard to be essential to establish the immunogenicity of attenuated P. pestis E.V. 76. When smeared over the surface of a blood-plate, the splenic pulp of this aethiops monkey yielded 11 colonies, which were phage-positive but nonpathogenic for mice upon intraperitoneal inoculation. According to Girard and Radaody-Ralarosy (1940), the persistence of P. pestis E.V. 76 in guinea pigs inoculated intraperitoneally with single doses of 1 billion (1 milliard) viable organisms never exceeded 13 days. The fact that in the highly susceptible Cercopithecus, P. pestis E.V. 76 51f was present in the heart blood, spleen, liver, and bone marrow, and persisted for at least 5 days longer than in guinea pigs following the inoculation of merely 16 million organisms, represents impressive evidence that the attenuated P. pestis E.V. 51f is pathogenic to a variable degree for the Ethiopian species of Cercopithecus. Infective capacity or "virulence" is demonstrated by the proliferation and persistence of the vaccine strain in the affected tissues. Among the host-effects, death was variable because the subhuman primates available were heterogeneous in their reactions. A graded dosage response was not measured. Even the low dosage of 160,000 viable E.V. 51f organisms induced marked clinical manifestations of disease. Since these reactions were not anticipated, no provisions had been made for measuring the host-effects more carefully except at autopsy. Visible lesions in the form of granulomas in the spleen and liver may be considered expressions of successful defense against a moderately virulent P. pestis. Of the parasite-effects, the most obvious were multiplication and survival of P. pestis in the tissues as an equivocal index of pathogenicity. Proliferation is a direct index of the extent and degree of parasitism. The effects of E.V. 51f in Cercopithecus were then compared with those observed in pathogenicity tests on Hartley guinea pigs. In these animals, the freeze-dried E.V. 51f in a total dosage of 15 billion, with 3 billion viable cells inoculated subcutaneously, caused no deaths but resulted in local lesions at the site of injection, regional lymphadenopathy, and granulomas in the spleen and liver. Thus, the isolate behaves like a moderately virulent microbe considered to constitute a safe vaccine for the immunization of man. Agar-grown (37°C) suspensions induce similar reactions in guinea pigs.

The antibody response was most vigorous in groups of Cercopitheci inoculated with the smaller dosages of the vaccine strains. The Fraction I antibody titers were definitely higher in the sera of animals vaccinated with 160,000 and 1,600,000 than with 160 million organisms. It is significant that on the 16th day after vaccination the mouse protection indices in these groups were already 1 or below. The serum dose of 0.5 ml of two Cercopitheci protected all mice against the standard infection. This single observation lends support to the claim of investigators who have studied the evidences of immunity produced with P. pestis E.V. 76 that by the 5th to 6th day after vaccination, definite signs of immunity may already be demonstrable in guinea pigs. The slight fluctuations in antibody titers observed are probably attributable to different degrees of illness in individual animals. The pathophysiologic damage caused by the endotoxin released during the course of the vaccination disease (if this is indeed the case) may in some unknown manner interfere with the antibody-producing mechanism. This phenomenon was negligible in the group comprised of animals receiving the lowest vaccine dosage (160,000 organisms). It is not surprising that recovery from a moderately severe P. pestis vaccine infection should confer solid protection against subcutaneous infection with 130,000 organisms or approximately 100 LD_{50} virulent P. pestis 195/P. All the vaccinated Cercopitheci survived a subcutaneous infection with 130,000 organisms or approximately 100 LD_{50}, while the unvaccinated controls inoculated with saline placebo died on the 4th to 6th day, with clinical signs of apathy, anorexia, fever, and positive blood culture on the 3rd day, disclosing classical pathology and bacteriological evidence of plague septicemia at autopsy. That the animals vaccinated with small doses of E.V. 51f effectively managed the challenge inoculation was an intriguing observation. No local lesions or lymphadenopathy developed; two had moderate rises in temperature on the 3rd day. In four of six monkeys vaccinated with large doses, local subcutaneous hemorrhages faintly outlined in the skin were observed, but these were of relatively short duration, in one animal accompanied by induration and spreading edema. Release of endotoxin and hypersensitivity to Fraction I are generally associated with such local reactions. Why these lesions appeared only in some Cercopitheci and not in others vaccinated with large doses is a subject for further study.

A mortality incidence of 3 from a group of 16 Cercopitheci vaccinated with a generally accepted, "safe," attenuated live plague vaccine elicits concern and deserves comment. The species of subhuman primate imported from Ethiopia has been superficially studied for susceptibility to virulent plague. Early deaths following subcutaneous virulent infections were characterized by ecchymotic hemorrhages within the skin extending subcutaneously with edema to the regional lymph nodes, which were invariably enlarged and hemorrhagic. All subcutaneous capillaries were distended and resembled the type of congestion commonly seen in mice and rats dead from acute plague infection. Enlargement of the spleen and degenerative changes in the liver and kidneys, clear pleural effusions, pulmonary edema, and occasional petechiae were all typical signs of acute plague invasion. In contrast, autopsies on Cercopitheci dead from generalized E.V. 51f septicemia revealed the usual capillary ramifications in the dehydrated subcutaneous tissues, but no hemorrhages, necrosis or edema. The lymph nodes draining the site of inoculation were enlarged (the size of Boston beans); they were grayish, slightly congested and mottled, not hemorrhagic. Spleens were invariably enlarged, in one instance five times the normal weight (42 gm). Granulomatous proliferations throughout the pulp, already present on the 5th and 6th day after vaccination, were more pronounced by the 18th day. Similar localized lesions appeared in the liver; they have not been observed in acute (6th day) virulent plague septicemia. The kidneys of the vaccinated animals invariably exhibited evidence of an acute parenchymatous degeneration similar to that of plague. Essentially the microscopic changes consisted of degeneration of the tubular epithelium with granular casts. Detailed examinations were not exhaustive, but the alterations clearly resembled those ascribed by Walker (1968) to pathologic effects of plague endotoxin. Varying (8 to 42 ml) amounts of clear transudate fluid in the thoracic cavities and pulmonary edema in both vaccinated and nonvaccinated subhuman primates may be attributed to permeability-increasing plague toxins acting on vascular endothelium. The difference in pathologic changes observed in Cercopitheci, which succumbed following vaccination, in contrast to those of fully virulent plague infection, was expressed by the absence of hemorrhages. Possibly this disparity may be due to varying concentration of the causative toxins or to other fluctuations in the toxins causing the vascular damage.

Further studies on Cercopithecus aethiops from Kenya were undertaken to answer the uppermost pertinent question: How injurious are attenuated living P. pestis E.V. vaccines to man?

Five groups each comprising four highly aggressive male Cercopitheci weighing 4.7 to 6.6 kg, held in individual cages after bleeding, were inoculated subcutaneously over the left femoral muscle with single doses (0.5 ml) of graded dilutions containing 218 million to 218 reconstituted freeze-dried living P. pestis E.V. 76 51f. Within 24 hours, the majority of the animals in groups vaccinated with the larger doses of vaccine were less active and more docile, eating sparingly. As the vaccine-induced illness progressed, fever and temperatures up to 41.2°C were recorded. Only the large doses resulted in local indurated swellings with or without involvement of local lymph nodes. The protocol stipulated the daily recording of WBC and quantitative blood cultures, but by the 6th day, continuous handling had definitely aggravated the illness. The collecting of blood became increasingly difficult because of peripheral venous collapse and rapid clotting, and examinations were discontinued. On the 6th day after vaccination with 2.18 million viable E.V. 51f, one Cercopithecus was found dead, with advanced rigor mortis. Autopsy of the dehydrated carcass revealed gross subcutaneous congestion of the skin, pleural effusions (28 and 40 ml), hypostatic pulmonary edema, dilatation of the heart cavities, splenic tumor, and the degenerative changes in liver and kidney previously encountered and interpreted as plague vaccine toxemia, without hemorrhagic-edematous infiltration at the vaccination site. The left inguinal lymph node, though enlarged to the size of a bean, was soft, edematous, and hyperemic but not hemorrhagic. The intestinal tract contained little greenish chyme and few rectal pellets. Blood cultures on the 4th and 5th days after vaccination yielded confluent colony growth of E.V. 51f on blood plates. However, isolation and quantitative estimation of the vaccine plague bacilli in cultures taken at autopsy were unsuccessful, since the organisms had been overgrown by enteric bacteria (E. coli, Proteus sp.). With the aid of selective media, isolation from the frozen tissues proved successful.

In rapid succession, 3 Cercopitheci on the 7th day and another 3 on the 8th day came to autopsy after clinical illness with and without fever, high WBC counts, and blood stream invasion of varying intensity. Gross pathologic findings were essentially the same as in the first victim of the E.V. 51f vaccine. The extent of enlargement of regional inguinal and iliac lymph nodes, the size of the spleen, and the degree of pulmonary hypostasis were subject to individual variations. Secondary terminal bacterial invasion from the intestinal tract complicated the quantitative estimation of vaccine plague bacilli in the viscera. However, it is significant that at autopsy the heart blood cultures of 4 animals yielded P. pestis E.V. on blood plate surfaces.

Two Cercopitheci each vaccinated with 218 million bacilli, and one inoculated with 2,180 E.V. 51f, all with proven bacteremia, continued to lose weight despite careful nursing; they died on the 15th, 20th and 28th day, respectively.

The group of 4 Cercopitheci each vaccinated with 218 viable organisms in an approximate total of 700 E.V. 51f appeared well and exhibited no local reactions, with only slight elevations in peripheral leukocytes. Blood cultures repeatedly proved sterile. On the 28th day after vaccination, their blood sera reacted strongly to the passive hemagglutination test (1:1024 to 1:8192) and similarly to the complement-fixing antibody (CF) test. The unexplained phenomenon observed in the first vaccination trial - i.e., animals vaccinated with small vaccine doses responded with slightly higher serum antibodies - was again evident.

The reactive behavior of this group is important because it indicates that a relatively small dose of live vaccine, provided it contains potentially invasive units, may effectively immunize *Cercopitheci* weighing from 5.1 to 6.6 kg without inducing local or systemic side-effects. Indirect evidence of protective efficacy has been confirmed: *Cercopitheci* surviving vaccination with E.V. 51f resisted subcutaneous plague in challenge inoculation of 100,000 *P. pestis* 195/P, a dose which proved fatal to nonvaccinated animals on the 3rd to 4th day after infection.

The outcome of the experiment served to emphasize the fact that Kenya *Cercopitheci* seem highly susceptible to the viable vaccine strain of *P. pestis* 51f and its toxins. The vaccination caused 10/20 (50 percent) deaths with severe side-effects, anorexia and pathophysiologic changes, thus preparing the host for secondary bacterial invasion from the intestinal tract. In retrospect, it is probable that hard-pellet food and frequent daily handling contributed to a high mortality rate. More elaborate care might have saved some of the experimental animals. One fact is paramount: no collection of animal hosts, however carefully selected, is so homogeneous in reaction to vaccination and infection that *all* individuals survive a given dose of microbes and *all* die after receiving a slightly larger dose. It is apparent that currently available Kenya *Cercopitheci* are too heterogeneous to be suitable for studies with small groups of animals. Plans have been negotiated to repeat the experiment in South Africa with *C. aethiops pygerythrus* using several hundred individuals, provided facilities can be procured.

It is regrettable that the blood cultures could not be continued, but the available data lend some support to the belief that it was not the inherent toxic content of the vaccine which caused the mortalities. During the first 6 days after vaccination, surviving *Cercopitheci* either yielded sterile blood cultures or else the bacteremia expressed in quantitative colony counts was of a low order. Thus it is believed that the fatal toxemia developed as a result of extensive proliferation of the vaccine plague strain and the continuous freeing of toxins which anchored to unaltered erythrocytes or endothelial cells, leading to that toxemia which is *sensu stricto* responsible for death or preparation of the host for secondary bacterial invasion. These views agree with those of R.V. Walker (*Current Topics in Microbiology and Immunology*, Vol. *41*, pp. 23-42, 1967), who, on the basis of his research, speculated on the nature and mechanism of plague intoxications. The experiment just reported may serve to guide future studies evaluating the safety and protective efficacy of the classical living P. pestis E.V. 76 vaccine. Since E.V. 51f was reconstituted from the freeze-dried state, it is apparent that lyophilization and storage at -20°C for at least 3 months have not damaged or reduced the protective potency of the isolate. Although a different subspecies of Cercopithecus was used as experimental host for testing the immunogenic potency of the freeze-dried preparation, the response was of the same magnitude as that observed in the Ethiopian animals which were given the aqueous suspension of the E.V. 51f isolate grown on agar at 37°C (Report 18, pp. 51-66). As anticipated from the antibody level (MPI of 5 and below) determined from the sera of the 10 surviving Cercopitheci, protection from subcutaneous challenge infection with 106,000 P. pestis 195/P on the 52nd day after initial vaccination was complete. Even the animals vaccinated with the small dose of 218 viable E.V. 51f effectively withstood the infection. Neither lymphadenopathy nor local lesions at the site of infection were apparent in any of the animals. In contrast, the two control monkeys succumbed to bubonic-septicemic plague on the 4th and 5th days, respectively. Here one observation deserves emphasis. The group of Cercopitheci inoculated with the smallest dose and clinically unaffected by vaccination responded with slightly higher antibodies than those given large doses. The appearance of complement-fixing antibodies in titers of >1:16 indicated that vaccination with the E.V. 51f had produced a type of infection indistinguishable from that induced by slightly virulent P. pestis, possessing (sic Girard) "residual virulence." It is reasonable to suspect that the smaller dose caused less injury to the immunity mechanism. Equally important is the fact that a single dose of 218 P. pestis E.V. 51f, capable of invasion, multiplication and persistence in the tissues, can confer solid protection against plague.

Confirmation of the observations made on two subspecies of Cercopithecus aethiops was obtained in South Africa. At the request of the principal investigator, the South African Institute for Medical Research in Johannesburg provided facilities to conduct vaccination experiments on the non-exportable South African vervet, Cercopithecus aethiops pygerythrus. Because of the dedication and generosity of Dr. M. Isaäcson (Department of Bacteriology) and Mr. A. F. Hallett (Medical Ecology Center) of the Institute, the essential findings of these studies have been made available. With their permission, important observations which they will publish are here briefly set forth and analyzed.

Groups of 10 Cercopitheci, with both sexes fairly equally distributed, weighing 4-6 kg each, were inoculated subcutaneously with dilutions of the reconstituted freeze-dried living E.V. 76 51f.

Vaccination fatalities occurred as follows:

		Total
Group I	Dose 1.009 x 10^8; death on days 4,5,and 6 (2)	4/10
Group II	Dose 1.009 x 10^6; death on days 5 and 11	2/10
Group III	Dose 1.009 x 10^5; death on days 6, 7, 11, 22	4/10
Group IV	Dose 1.009 x 10^3; death on days 8 and 28	2/10
Group V	Dose 101; death on day 9	1/10

Summary: 13/50 or 27 percent vaccination fatalities.

The highest (40 percent) fatalities occurred in groups inoculated with 100 million and 100,000 organisms. Susceptibility is heterogeneous; one death was even caused by 100 viable E.V. 51f.

According to sketchy reports, local and systemic reactions following vaccine inoculations were quite similar to those observed in Ethiopian and Kenya *Cercopitheci*. The erythemas and indurations at inoculation sites disappeared within 4-5 days, but the regional (inguinal) lymph nodes had enlarged in 2-3 days and persisted as marked, firm lymphadenopathies for up to 25 days. Fever was recorded irregularly in every animal; WBC were elevated up to 22,000 (even to 131,200 in one *Cercopithecus* shortly before its death on the 7th day). Blood cultures were obtained as early as the 2nd day and varied from a few to diffuse, uncountable colonies per ml on blood agar surfaces. Even animals which survived had temporary blood-stream invasion. Gross and microscopic lesions at autopsy were identical to those observed in other experimental series: local inguinal lymphadenopathy, with little or no hemorrhage extending to iliac and para-aortic nodes; subcutaneous congestion; pleural and pericardial slightly blood-tinged fluids; splenic tumor; parenchymatous degeneration of kidney and liver with microscopic necroses; adrenal hemorrhages; pulmonary congestion and edema. Late deaths were accompanied by emaciation and terminal enteritis (*Proteus mirabilis* and *P. rettgeri*). *P. pestis* was invariably isolated from the heart blood and lymph nodes, thus confirming the post mortem diagnosis of bubonic-septicemic plague.

The antibody response of survivors was also similar to that observed in the Kenya species. HA titers varied from 1:4 on the 2nd day and 1:128 on the 8th to 1:4096 and even 1:32,748 on the 28th day, while the MPI of 9 animals varied from 1 (2) to 2, 3 (4), 6 and 11 on the 28th day.

Since the animal quarters were urgently needed for other studies, the project had to be terminated by the 30th day; and since space in the isolation laboratory was limited, only *10 Cercopitheci* -- 2 selected at random from each group -- were reserved for protection tests by experimental infection. They were inoculated subcutaneously on the 166th day (more than 5 months) after vaccination with 8,770 virulent *P. pestis* (F357 Lesotho, former Basuto, 1968 outbreak). The PD50 of the challenge dose had not been determined for mice. Three of the challenged animals succumbed to septicemic plague:

Group I	100 million *P. pestis*	HA 1:32, MPI *17*, WBC 23,000 (day 4). Blood culture negative (4); death on 8th day.
Group II	10,000 *P. pestis*	HA 1:128, MPI *17*, WBC 21,900 (day 8). Blood culture 150 cols/ml (8); death on 12th day.
Group IV	1,000 *P. pestis*	HA 1:64, MPI 15, WBC 13,500 (day 4). Blood culture negative (4); death on 10th day.
Unvaccinated control 1		MPI *14*, WBC 31,000 (day 4). Blood culture *200 cols/ml (day 4)*; death on 6th day.
Unvaccinated control 2		MPI *14*, WBC 10,800. Blood culture negative (2nd day); death on 7th day.

One of the 2 *Cercopitheci* in Group V developed a local suppurating axillary bubo as evidence of relative resistance, but recovered. The other survivors of the experimental infection appeared well and revealed no local reactions or lymphadenitis at the site of the *P. pestis* inoculation. HA titers and MPI indices determined from sera collected shortly before challenge had all declined. Three animals which were not protected had MPI of 17 (2) and 15 and HA of 1:32 to 1:128. Only 2 of the 7 monkeys proven immune had MPI of 5. Regrettably, unexpected circumstances limited experimental challenge infection to 10 (or only 27 percent) of the survivors of this vaccination trial. Thus meaningful survival percentages could not be calculated. It is indeed surprising that the living, highly invasive, partially virulent E.V. 51f, which conferred absolute protection with as few as 218 viable organisms upon other *Cercopithecus aethiops* species for at least 52 days, apparently failed to immunize 3 South African species. Judged by the antibody response on the 28th day after vaccination, immunity was solid (MPI all below 5). The challenge infection dose was 1/15 the amount used in preceding experiments on African green monkeys. Originally the experimental protocol called for determination of the susceptibility of the C. pygerythrus species on groups of 10 animals, but since suitable isolation quarters were not available, only 2 (previously nonvaccinated) animals were infected. They succumbed, with anatomic and bacteriologic findings of acute septicemic plague, on the 6th and 7th day, respectively, approximately at the same phase of the disease as Ethiopian Cercopitheci fatally infected with 200 to 20,000 P. pestis 195/P (Report 18, p. 46). It may be tentatively assumed that the susceptibility of South African vervets is identical to that of the Ethiopian species. The immediate response to vaccination was excellent, as evidenced by low mouse protection indices on the 28th day. The factors responsible for the loss of protection in 3/10 animals by the end of the 5th month remain unknown (Report 19, pp. 11-14).

In retrospect, it is recalled that the enhanced virulence and pathogenicity of the live E.V. 51f passage isolate was first demonstrated in extensive experiments on Cercopithecus aethiops species -- not by safety tests on guinea pigs. Subsequently the nefarious side effects of this vaccine were confirmed in a single test on langurs.

Several vaccination experiments to evaluate other live plague strains were invariably made on a comparative basis, using 3 species of nonhuman primates. Six Kenya vervet monkeys, each intradermally inoculated with 26,800,000 E.V. 76 Paris, had local skin reactions which were less marked than those in langurs: pale nodules surrounded by faintly reddish, slightly indurated areas were replaced by eschars or scaly desquamation of the readily folding skin by the 8th day after vaccination. Enlargement of the left inguinal lymph nodes was moderate in comparison to that in langurs; evolution to prominent nodes from the 5th to 16th day, receding to 1.8-cm swellings by the 27th day, differed only in intensity from such reactions in langurs. Four of the 6 vervets exhibited temporary elevations in temperature, possibly the result of stress during capture, particularly in those which remained aggressive during the course of daily examinations. Although all were eating well, they had leukocytosis and 3 animals which were bled on the day they disclosed body temperatures above 40°C yielded a few colonies obtained on blood plates or by enrichment in broth culture on the 2nd or 5th day. Lymph node aspirates from biopsy specimens taken on the 5th day yielded the same results as those recorded for langurs. A large node when punctured revealed only a few P. pestis (Paris). Antibody response to living E.V. 76 Paris 1 month after vaccination was equally as vigorous as in langurs. The decline in titers after 2 months had elapsed was definite, both in hemagglutinin and mouse protective antibodies, and may have been responsible for the apparent failure of the immune mechanism to protect completely. The subcutaneous challenge infection, produced with a smaller dose than that used in langurs, was not innocuous; it caused local inflammatory nodules with edema. The acute, necrotizing, small abscess development led to fistula formation. Discharge from one such abscess yielded virulent P. pestis on the 9th day. By the 14th day after vaccination, the non-draining nodules had been absorbed. Careful examinations invariably failed to disclose palpable enlarged lymph nodes. No elevations in body temperature and no general symptoms were recorded. Compared with those inoculated with Fraction I or M_{23}, the animals were active and ate well. Fatal septicemic bubonic plague on the 3rd to 4th day in the nonvaccinated control Cercopitheci was accompanied by widespread edema over the abdomen and the tissues surrounding primary bubonic plague lesions in the axillary lymph nodes. The protection conferred by live vaccine E.V. 76 Paris was not absolute. Virulent plague bacilli were apparently localized and rendered non-invasive, but the release of toxic antigens caused severe local inflammation and even abscesses wherein the bacilli remained viable for at least 9 days (Report 19, pp. 35-36). The antibody response in Cercopitheci is equally as vigorous as in langurs. However, in the limited experiments conducted, it became apparent that decline in titers is slightly more marked in the African vervet species and may be partially responsible for the incomplete protectiveness of the immune mechanism.

Local reactions in a group of macaques (each weighing 3.8 to 5.9 kg) previously used in malaria experiments and not in prime condition were confined to pinhead-sized, pink nodules, which were reduced to hairless areas by the 8th day after vaccination. Regional adenopathy in the form of firm but movable nodes (maximum 2.9 x 2.0 cm) was similar to that observed in this species inoculated with the Fraction-I-negative P. pestis M_{23}. In each case, biopsy of the large left inguinal lymph node at the time the right node became palpable proved fruitless. No temperature elevations or leukocytosis were recorded. Nonspecific diarrhea developed in 2 animals; one yielded a positive blood culture 2 days before death. Autopsy findings were atypical for plague and apparently complicated by nonspecific gastroenteritis and a chronic pneumonic abscess due to Klebsiella sp. Nonetheless, E.V. 76 Paris was isolated from the heart blood, left and right inguinal lymph nodes, left enlarged iliac lymph node, spleen, liver and bile. Correlation between HA, CF and MPI on the 28th day after vaccination was definite. The absence of in vitro antibodies (HA and CF) but persistence of in vivo protective antibodies (MPI 2 to 8) 4 months after vaccination remains unexplained. Solid protection against severe intraperitoneal infection which proved fatal to controls on the 4th and 5th days, and the recall of HA titers to 16,384 and CF antibody titers to 1:32 one month after challenge, confirm the finding in langurs and African vervets that the E.V. 76 Paris may effectively serve as a highly protective plague vaccine. However, the experiments on these very susceptible nonhuman primates indicate that this isolate in moderately large dosage is capable of producing unpleasant local and systemic side effects (Report 20, p. 12).

In view of this more recent information, it is recommended (1) that healthy, readily accessible, well conditioned African vervets may be used as a substitute for langurs; and (2) that the guinea pig so highly regarded for the past 40 years as the animal of choice to test the "safety" of a live attenuated plague vaccine, be replaced by one of the very susceptible nonhuman primates.

II. Standardized Quantitative Potency Tests.

Mere selection of an experimental animal suitable for the evaluation of the immunogenicity of a vaccine or antigen by no means solves the majority of the problems involved. Quantitative studies must be conducted with large series of animals. The test dose of virulent P. pestis for challenge infections must be carefully established. P. pestis bacilli grown in the spleens of rats or guinea pigs, as practiced by early workers, especially at the Haffkine Institute, vary considerably in number; they have been replaced by standardized broth cultures (Sokhey and Maurice (1934, 1935)). The method of these two investigators consists in determining the minimum quantity of a plague vaccine or antigen that would protect a mouse against a standard infective dose consisting of 1000 MLD (2000-4000 bacilli) of a wild plague strain. Maximum virulence is maintained by frequent passages through guinea pigs, and the bacilli are stored as blood agar slant cultures at ±4°C. The principle of standardized potency tests has been successfully practiced on guinea pigs and monkeys. The infective dose is administered to mice on the 7th day and to guinea pigs or monkeys 21-28 days after the second inoculation of the vaccine being tested. The LD_{50} chosen must be such that all the animals die. The antigen or vaccine to be tested is inoculated subcutaneously or intraperitoneally in 5 to 8 different amounts, using series of not less than 10 animals for each dilution. The Reed-Muench formula is used for expressing the results of protection tests on mice and guinea pigs. Such potency assays on nonhuman primates are prohibitive in cost; consequently they are performed with mice or less frequently, guinea pigs. For the sake of consistent uniformity, the infective challenge dose may be partly standardized: A loopful of stock culture from the blood agar slant is sown in 5 ml of a broth consistent in uniform composition and noted for good growth-promoting qualities. After incubation for 18-24 hours at 37°C, 0.5 ml of this culture is inoculated into another 5 ml of the same broth and identically cultured for 18 hours. The culture is then diluted out into 1 percent peptone water. Experience has demonstrated that a 1:10,000 dilution of the culture will give a total count of 2000-4000 organisms per 0.2 ml, which is the recommended dose for mice. Subsequently the count is checked by a surface plate count of 0.2 ml of 5 percent blood agar, using a 1:1,000,000 dilution. Guinea pigs or monkeys receive a lower dilution or 1000 to 10,000 times higher doses, respectively. In many ways, this animal experiment does not reflect the natural conditions of infection because it is usually impossible to reproduce consistently the infectious disease with regard to portal of entry and infective dose.

III. Measurements of Antibodies in Peripheral Blood of Vaccinated Animals and Man.

For years experiments on immunization against plague have been guided by the prevailing concept that if a live or killed whole vaccine or an extracted antigen fraction presented good presumptive evidence of efficacy in mice and guinea pigs, and occasionally in nonhuman primates, it could be used in an effort to minimize the incidence of plague in human subjects. There is ample evidence that most investigators have restricted their observations on immunity to the percentage of animals surviving after challenge. In several instances, vaccines which proved highly protective to guinea pigs and apparently nontoxic to small laboratory animals, when tested on man caused extremely unpleasant side effects. There is general agreement that proven meaningful valid potency assays in laboratory animals must be correlated with properly conducted clinical trials on man. Selecting a vaccination site, mode of administration and schedule is not enough: The complete trial should include some test(s) designed to measure the degree of resistance or protection conferred upon man by a plague vaccine proven efficacious in assays on animals. Measurements of antibodies in peripheral blood have received considerable attention since Jawetz and Meyer (1944) developed the technique. Despite the warning that "conclusions on the immunogenicity of vaccines for man deduced from serological response to them should be held with reservation" (Burrows 1963), the following *in vitro* and *in vivo* serological tests have been studied and evaluated.

(A). Passive Mouse Protection Test (MPI): A modification of the mouse protection test used to standardize the protective potency of curative antiplague rabbit gamma globulin preparations developed during World War II was devised to measure the protective antibodies in animals and humans recovered from plague and following inoculations with vaccines. The procedure developed by the G. W. Hooper Foundation Laboratory (Meyer and Foster 1948) and during the past 24 years practiced by the junior investigator (L. E. Foster) is as follows: At least 10 mice are each given an intravenous injection of 0.5 ml of the undiluted (or diluted 1:2 if the protection is strong) serum to be tested, immediately followed by 100 MLD of a highly virulent culture of P. pestis subcutaneously. The mice are observed for a time sufficient to allow complete development of infection in this species -- about 14 days. Sera collected after recovery from plague infection afford considerable protection to mice, as indicated by lower fatality rates and longer extension of the average death time. The differences, first in the percentage of animals that die, and second in the length of survival time, are not especially meaningful when considered separately, but the two factors together yield significant data. In order to facilitate comparison by expressing these two factors in a single figure, the percentage of mice that die is divided by the average day of death. The quotient is designated the "Mouse Protection Index" (MPI).

The results of the MPI test are reproducible and significant when in the index range of 1 to 5. With some sera derived from macaques inoculated with some vaccine preparations, the test proved insufficiently sensitive to measure indirectly the state of immunity.

It was suggested that the MPI test be compared with the quantitative agglutination test. The primary theoretical objection to any bacterial agglutination test is that it will measure total antibody, not protective antibody alone. Used as an agglutinating antigen, the whole plague organism is composed of two soluble, highly immunogenic proteins, easily removed from the whole organism by simply washing and centrifugation. The mean value of loss of these soluble antigens (Fraction I and "murine toxin") in 5 trial experiments was 39.4 percent of total bacterial nitrogen. The highest and lowest losses were 79 percent to 17 percent, respectively. Fraction I is the most protective antigen and is very soluble. In its soluble state it may very readily be present in excessive amounts in this type of test and be "out of phase" with an agglutination test otherwise showing a maximal antibody N adsorption value.

These technical difficulties were resolved as much as possible by using for a control the bacterial N obtained by centrifugation of the organisms in the presence of normal serum, followed by washing with saline.

For the agglutination test, 0.1 ml of a suspension of 195/P (498-500 gammas dry wt/ml) containing 8 to 11 µg antibody N is added to 0.02-1.0 ml serum. The mixture is incubated in test tubes for 1 hour at 37°C, then overnight at 4°C. The resulting precipitate in each tube is washed 3 times in an angle-head International PR-8 refrigerated centrifuge at 3,000 rpm for 1 hour at 4°C. Precipitates are next digested in boiling sulfuric acid in the original test tubes for 1 hour. Nessler's reagent is then added directly to the digested material, and the amount of color developed is determined in a Coleman Universal Spectrophotometer at 500 mμ. A standard protein curve is made each time, with varying amounts of bovine serum albumin.

The quantitative bacterial agglutination method has been compared with the passive mouse protection index (MPI), using three human serum pools with different MPI:

	Antibody N/ml(μg)	Ratio of fixations	MPI	Survivor/ Total	Ratio of indices
Group I (Good protection)	36.5	2.7	2	8/10	6
Group II	19.5	1.4	8	1/10	1.5
Group III (Poor protection)	13.5	1.0	12	0/10	1.0

The relative ratios of increase in terms of antibody N/ml fixed and MPI in Groups I and III show that the MPI was at least twice as sensitive as the quantitative agglutination test in detecting protective antibody increases. The more sensitive MPI would indicate "poor" or "good" protection with greater precision and allow for a larger margin of error than the agglutination test. As usual, an MPI below 5 or 10 indicated protection.

The MPI, based on the passive serum protection of mice, evaluates the in vivo protection afforded by plague antibodies. The HA and CF tests using Fraction I as a detecting antigen semi-quantitatively evaluate antibodies to the major protective antigen itself. The two methods seldom yield conflicting results. The quantitative bacterial agglutination test afforded no marked advantage nor superior degree of differentiation over the other tests.

(B). Toxin Neutralization: Toxin of the deoxycholate type prepared according to method of Goodner et al. (1954) in serial 4-fold dilution was mixed with an equal volume of approximately diluted serum to be tested and the mixture was held at 37°C for 1 hour prior to injection of 1.0 ml amounts into the peritoneal cavities of 8 mice. The test demonstrated that the toxin is antigenic and that patients convalescing from pneumonic plague developed what appeared to be toxin-neutralizing substances (McCrumb et al. 1955).

(C). Bacterial Agglutination Test: Repeatedly washed suspensions of a highly virulent formalin-killed strain of *P. pestis* which produces more than 15 mg percent Fraction I are used as antigens in the customary macroscopic tube test (Jawetz and Meyer 1944). This was replaced by the passive hemagglutination test.

(D). Complement Fixation Test: According to the procedure developed by Chen *et al.* (1952), Fraction I is dissolved in physiologic saline in a proportion of 2 mg per 1 ml (stock solution), and for routine tests is further diluted 1:500. Two-fold dilutions of inactivated sera to be tested (0.2 ml) are placed in a series of tubes. A 1:500 dilution of stock Fraction I antigen solution (0.2 ml) and 0.2 ml of complement (2 units) are then added to the tubes, which are shaken, incubated for 4 hours at 4°C (refrigerator), and then kept for 1 hour at 37°C (water bath). Blood cells (0.4 ml) sensitized by appropriate amounts of hemolysin 15 minutes before use are added to each tube. After incubation at 37°C for 30 minutes, preliminary readings may be made. Holding the racks or microtitration trays in the refrigerator overnight results in more accurate final recording of the reactions.

(E). Passive Hemagglutination Test: The technique developed by Chen and Meyer (1954 and 1966, WHO), adapted to the microtechnique by Sever (1962), has been recommended by the WHO Expert Committee on Plague (1970), Rep. Ser. 447. The procedure is as follows:

(1) Reagents: All reagents used in the tests must be of the highest quality. Sterile, non-pyrogenic water and sodium chloride for injection are used throughout as saline diluent or as diluent for the preparation of any reagent. Tannic acid for tanning sheep erythrocytes must be of reagent grade and protected from oxidation.

Normal rabbit sera employed as diluent in the tests must be obtained from rabbits free from the risk of exposure to any of the *Pasteurella*. The sera are absorbed with sheep erythrocytes, as described by Chen and Meyer (1954), prior to the hemagglutination test.

(2) Equipment: All centrifugation is performed at 5°C in a refrigerated centrifuge. For all hemagglutination tests, suitable equipment, such as the "microtiter" equipment described by Sever (1962), is required.

(3) Collection and tanning of sheep erythrocytes: Appropriate precautions must be taken to ascertain that the erythrocytes used in the tests are obtained from suitable sheep. The cells are stored in Alsever's solution at 5°C. For use, the cells in Alsever's solution are washed (1500 rpm) 3 times in normal saline and then

suspended in normal saline to give a 2.5 percent concentration. Next an equal volume of freshly-prepared tannic acid solution (1:20,000 w/v) is added. The resulting suspension is thoroughly mixed and allowed to stand for 15 minutes in a water-bath at 37°C, with frequent shaking. The tanned erythrocytes are then washed (1500 rpm for 3 minutes) once in saline (pH 6.5) and resuspended to a 2.5 percent concentration in saline (pH 6.5). This suspension is divided into 2 aliquots.

(4) Sensitization of tanned erythrocytes with Fraction I antigen of the plague bacillus: A 200 µg/ml solution of Fraction I is prepared in normal saline (pH 7.0). An equal volume of the Fraction I solution is added to 1 aliquot of the tanned erythrocytes, and the mixture is kept at room temperature (about 25°C) for 15 minutes, with frequent mixing. This suspension is then centrifuged (1500 rpm) and the supernatant is discarded. The cells are washed twice (1500 rpm for 3 minutes) with 1:100 normal rabbit serum in normal saline (pH 6.5). The Fraction I sensitized erythrocytes are then resuspended in a 1 percent concentration in 1:100 normal rabbit serum in saline (pH 6.5).

(5) Preparation of tanned erythrocytes for control antigens: An equal amount of 1:100 normal rabbit serum in normal saline (pH 6.5) is added to the second aliquot of tanned erythrocytes, which is then treated identically to the preparation sensitized with Fraction I. This serves as a control antigen for nonspecific agglutination.

(6) Diluent for antibody inhibition test: This diluent consists of a 25 µg/ml solution of Fraction I in 1:100 normal rabbit serum in normal saline (pH 6.5).

(7) Test procedure:

(a) All test sera are inactivated at 56°C for 30 minutes and absorbed with the sheep erythrocytes used to prepare the sensitized and control antigens. Each series of tests must include 2 known-positive control sera and 2 known negative control sera.

(b) Each serum is tested in triplicate.

(c) For each serum that is to be tested, diluents are added to 3 rows of the microtiter "U" plate as follows:

(1) The volume of each diluent to be added is determined by the dilution scheme selected. A twofold dilution scheme is recommended.

(2) A 1:100 dilution of normal rabbit serum in normal saline (pH 6.5) is added to each well of a microtiter "U" plate in rows 1 and 2.

(3) The diluent for the antibody inhibition test is added to each well of the "U" plate in row 3.

(d) Identical volumes of the test serum are added to the first well of the "U" plate in rows 1, 2, and 3. The sera are then progressively diluted in the following wells by means of microtiter loops.

(e) Tanned control cells are added to the serum dilutions in row 1.

(f) Tanned erythrocytes sensitized with Fraction I antigen are added to rows 2 and 3.

(g) Plates are sealed with plastic tape, left overnight at room temperature on a level surface, and read with a microtiter mirror the next morning.

(8) Interpretation of results: A compact button with smooth edges in the center of the well represents a negative reaction. Positive reactions are characterized by uneven distribution of erythrocytes over the bottom of the wells, and the margins of the cell patterns are very uneven and rough. An acceptable test is controlled by the reactions observed in the known positive and negative sera. The known negative sera must be entirely negative, and the titer of the known positive sera must agree with one dilution with the titers observed with the same sera in previous tests. There must be no agglutination in the dilutions of the unknown sera tested with the control cells, or the reaction with tanned erythrocytes sensitized with Fraction I must be at least 4 dilutions greater before the reaction can be considered at all specific. Specific reactions are confirmed by the Fraction I antibody inhibition test: sera that are positive in the dilution series tested against tanned erythrocytes sensitized with Fraction I are negative when diluted in diluent that incorporates a 25 µg/ml concentration of Fraction I.

(9) Endpoint of titration: The endpoint of titration is considered to be the last clear-cut 4++++ reaction.

(10) Collection of blood: Blood for the tests is collected in tubes. The practicability of collecting blood samples on filter paper is being investigated.

(F). Hemagglutination Method to Measure "Murine" Antitoxin: One volume of 2.5 percent suspension tannic-acid-treated human Type O erythrocytes is added to 4 volumes of buffered saline (pH 6.4) containing the highly purified antigen in a concentration of 8 µg/ml. Excess antigen is removed from the cells by sedimentation and resuspension in fresh saline solution containing 0.4 percent rabbit serum. The toxin-treated cells are used in the same manner as that recommended in the procedure to test human and animal sera for Fraction I antibodies (Landy and Trapani 1954). Studies by Payne et al. (1956) conducted on sera derived from Malagasy demonstrated that nonvaccinated persons who have recovered from pneumonic or bubonic plague usually possess appreciable levels of antibody against Fraction I for months or years, but rarely maintain significant amounts of antitoxin. In contrast, those who had been vaccinated prior to infection usually have considerable amounts of both types of antibody for months or years after recovery (Payne et al. 1956).

IV. Significance of Serum Antibodies in Plague.

The fact that rabbits and mice inoculated with live attenuated P. pestis (A1122) or formalin-killed virulent P. pestis (H-1-4 and 106) developed agglutinating and in vitro protective antibodies (Jawetz and Meyer 1944) suggested that studies be undertaken along the following lines: (1) measurements of antibodies in the sera of animals and men recovered from plague, and (2) titrations of sera from animals and men immunized against plague and thus affording some guidelines for vaccination procedures and types of vaccines to be employed. These extensive studies yielded the following results:

(A). Human subjects: The sera of 6 men who had recovered from plague invariably fixed complement in titers varying from 1:32 to 1:512. The bacterial agglutination titer rarely exceeded 1:160, while the mouse protective index was below 10 (below 5 in two) -- average 5.6. Collected on the 21st day after the onset of disease (18 days after fever had subsided), the serum of a patient who had recovered from pneumonic plague with terramycin therapy yielded an agglutination titer of 1:32, complement fixation of 1:64 +++, and mouse protection index of 1.5. After 4 months (114 days) the titer declined to an MPI of 8. Single or paired sera from 19 patients who had survived bubonic or pneumonic plague with chemotherapy became available during a study in Madagascar. In vitro antibodies appeared by the 11th day of disease; the titers rose rapidly and MPI indices declined, so that by the 15th to the 18th day antibodies sometimes reached such concentrations that the human serum, diluted 1:10 and inoculated intravenously in the standard dose of 0.5 ml, protected all of 10 mice. The protective power of one serum was expressed by an MPI of 16 recorded on the 9th day. This had changed to 6 on the 13th day and to 0 by the 18th day, accompanied by complement-fixing antibodies of 1:512, hemagglutinin 1:10,240 and bacterial agglutination titers of 1:256. Late convalescence sera demonstrated that no absolute CF/MPI concordance exists, but that some parallelism in respect to HA and CF antibody titers is discernible. These observations established for the first time that recovery from plague, which is customarily followed by a relative moderate immunity to reinfection, is reflected in the antibody level expressed in the mouse protection index (Report 4, and McCrumb *et al*. 1955).

During an investigation of plague outbreaks in South Africa (Ovamboland 1962-63), the sera (collected 60-95 days after onset) of 18 patients revealed hemagglutination titers ranging from 1:32 to 1:32,768 (average 1:7839) and CF titers from 0 to 64 (average 14, except in one individual with a chronic infection, who had a titer of 1:256). Within 10-11 months the titers dropped significantly to one-half to one-sixteenth of their original level; some sera yielded no titers. Following bubonic infection, particularly in children, specific antibodies may drop markedly within 3 months and may disappear in 1 to 2 years. The Ovambo studies (Report 14, p. 32) confirmed previous observations by Payne and his associates (1956) that patients recovered from plague develop hemagglutination Fraction I antibodies which may persist at significant levels (1:160) for 3 years. The titers are higher and are maintained for longer periods in individual human subjects vaccinated prior to contracting the disease. The majority of persons who had previously received live plague vaccine had an antibody response following re-inoculation. It was concluded that the aim of all procedures in the active immunization of man against plague must be to select such vaccines or antigens and to inoculate them in such doses and schedules as will ensure the maximum production of hemagglutinins and passive mouse protection antibodies expressed in indices below 10, preferably below 5.

(B). *Nonhuman primates*:

(1) Sera collected from *Macaca mulatta* on the 14th to the 30th day after they had recovered from plague (bubonic or pneumonic) yielded hemagglutinins in titers of 1:32 to 1:4096; CF antibodies of 1:4-1:32, and mouse protection indices below the toxin-antitoxin neutralization indices varied from 2.3 to 22. Immunity tested by reinfection is solid for up to 3 months. When the interval between primary infection and intratracheal re-infection with (2.7×10^4) *P. pestis* was approximately 8 months, some individual monkeys succumbed to delayed pulmonary plague. Between the 8th and 10th month, immunity deteriorated and less than 50 percent (7/14) of

the recovered macaques survived 5.1 x 10^6 *P. pestis*. Only monkeys with HA titers of 1:256 or higher survived intratracheal challenge. Eleven animals each given 1 mg of Fraction I during the 6th month had responded with HA titers of 1:1024 to 4096 26 days before challenge and had remained well. The only victim of fatal reinfection had a titer of 1:64. In a series of 30 macaques (10 inoculated with Fraction I in 2 doses), only 2 developed mouse protective antibodies below 5 and resisted subcutaneous infection. The sera of 20 animals inoculated with a formalin-killed vaccine of low potency in 2 doses failed to yield significant antibody levels. Ten monkeys were each challenged with 10,000 *P. pestis* intratracheally; none survived the infection, although the pulmonary disease was delayed. The third group of 9 animals was held for 233 days until hemagglutinins had dropped in some instances to the level of 0 and all mouse protection indices had declined to normal levels (average 17). Two booster inoculations with the same low-potency vaccine promptly stimulated hemagglutinins to titers of 1:64 to 1024 and mouse protection indices to 1 to 7 (average 4). *All survived* intratracheal infection with 12,000 *P. pestis* 195/P. Resistance was definitely reflected by the level of serum antibodies.

(2) *Cynomolgus irus*: Similar experiments with this species demonstrated that only formalin-killed vaccine in Drakeol-Arlacel oil mixture is capable of promoting antibodies in titers that apparently confer solid protection (Reports 5 and 6).

(3) Langurs (*Presbytis entellus*): Humoral antibody levels in survivors 6 weeks after *P. pestis* infection consisted of CF titers of 8 to 128; HA, 256 to 8192; and MPI, 8 to 2. As observed in human subjects (Payne *et al*. 1956), the antibody response to subcutaneous infection (125,000 *P. pestis*) of survivors previously immunized with USP vaccine or with crude *Pasteurella pestis* toxic antigen was significantly more vigorous than in individuals not previously vaccinated and merely surviving infections (Chen and Meyer 1965). CF titers of 1:64 on the 28th day but only 1:4 by the 66th day were accompanied by HA titers of 2048 to 131,000 (Report 18). Upon recovery from subcutaneous plague infection, langurs injected with a live plague vaccine (E.V. WR-S) had higher HA and CF antibodies by the 36th day than they had during the vaccination period (Report 19).

(4) African vervets (*Cercopithecus aethiops sp*.): Survivors of vaccination experiments with live attenuated *P. pestis* E.V. 76 (51f, Paris, and Paris f), even in small doses, responded with hemagglutinin titers of 512 to 8192, complement fixing titers of 4 to 64, and mouse protection indices of 5 to oo. Invariably they

resisted severe subcutaneous infections. The Fraction I negative isolate M_{23} induced low HA titers, corresponding MP indices above 10, and no protection (Report 18).

(C). Guinea pigs: The blood sera of guinea pigs (heterogenous or Hartley inbred stock) which had recovered by the 30th to 60th day after plague infection in the course of protection experiments have been extensively and systematically tested for antibodies. Records on 138 guinea pigs disclosed the following significant average figures:

Bacterial agglutinins:	1:120
Hemagglutinins:	1:905
Complement fixing antibodies:	1:95
MP Index:	4

The significance of serum antibodies in guinea pigs inoculated with a variety of killed vaccines and antigens is routinely assessed on at least 4 animals exsanguinated on the same day that the degree of protection is measured by challenge infection. With the use of bacterial agglutination test alone, correlation between antibodies and protection has rarely been demonstrated. As more sensitive serologic methods were developed, it became possible to detect circulating antibodies, even in groups with only partial protection. Hemagglutinins (up to 1:32) were often detected when the results of other tests proved negative and no resistance was demonstrable. It was noted that in guinea pigs high hemagglutinin titers were invariably accompanied by complement fixing antibodies, low MP indices and a high order of protection (Spivak et al. 1958; Chen et al. 1964). The relationship between demonstrable antibodies and resistance to challenge in the guinea pig has been questioned, since this animal proved unsuitable for the testing of plague antisera. The results of experiments by Spivak et al. (1958) established the fact that the guinea pig is indeed more refractory to passive immunization than the mouse. Hyperimmune sera yielded some evidence of protection -- a higher percentage of survivors and extended survival time. In particular, rabbit antiplague globulins in dosages of 2500 mouse protection units, administered intraperitoneally 24 hours prior to subcutaneous infection with 10^6 virulent P. pestis, modified the course of the disease and resulted in the survival of 70 to 90 percent of the animals.

(D). Mice: Pools of 5 sera collected from mice on the 20th day after infection yielded average hemagglutinin titers of 1:512 to 1024 and CF of 1:8 to 1:16; however, some pools were nonreactive in the CF test. The high protective efficacy of a formalin-killed vaccine in adjuvant, which protected 78/80 or 97.5 percent of the

TABLE 1

IMMUNOGENIC RESPONSE OF MICE TO CUTTER KILLED PLAGUE VACCINE.

		Serology, Day of Challenge				
Interval After Immunization* week	Adjuvant	Complement Fixation	Hemagglutination	Bacterial Agglutination	MPI	Result of Challenge S/T**
1	0	0	0	0	9.8	19/20
2	0	0	128	0	8.2	18/19
3	0	0	64	0	10.8	16/20
3	+	16	2,048	16	4.7	20/20
4	0	0	16	0	11.4	10/20
4	+	8	2,048	8	1.0	20/20
5	+	16	2,048	32	oo	17/20
6	+	16	2,048	16	1.6	19/20

*With 20 μg Army-Type Vaccine, given in 2 doses, 7 days apart.

Challenge: Time - 1 to 10 weeks
Dose - 1,600 to 4,000 *P. pestis* 195/P
Route - Subcutaneous

**Survivors/ Total

inoculated animals, was reflected by significant antibody levels. Serum pools from 20 mice bled on the day of challenge yielded the following titers: Bacterial agglutinins, 1:8-1:32; hemagglutinins, 1:2048; CF, 0 to 1:2; MPI, 5, 1 and oo. High degrees of protection were thus correlated with high antibody levels. In other experiments, absence or a low level of antibodies was invariably correlated with absence of infection or weak resistance to it (Reports 7 and 12). This behavior is strikingly illustrated by the records of an experiment performed to determine the antibody response and protection afforded by formalin-killed virulent P. pestis vaccine (Cutter).

The pattern of antibody levels and degree of protection after vaccination with killed whole P. pestis suspension vaccine with and without adjuvant is compatible with current immunological concepts. The appearance of CF antibodies when the antigen persists in the tissues is particularly significant. The state of protection is accurately indicated by the level of the MPI, which was determined in pools of serum from 10 mice.

(E). Rabbit: This species, an excellent producer of antibodies in high titers, is most useful in the study of specific plague antigens (Fraction I, V and W; murine and endotoxin); it acquires variable individual resistance to experimental infection by relatively large doses of virulent P. pestis. Intraperitoneal infection with one billion P. pestis 195/P was fatal to 4/5 rabbits weighing 5.5 to 6 kg. Young rabbits around 12 weeks old, ordinarily used for experiments, are fairly uniformly susceptible. Immunization experiments usually extend from 3 to 6 months; by that time, some of the rabbits have acquired age resistance, thus rendering evaluation of the results of immunization impossible.

The most consistent infections were achieved with the intercostal injection of virulent P. pestis. This is of course a highly artificial means of introducing the organisms, but it does circumvent the effective subcutaneous and intraperitoneal defense mechanism of older rabbits.

The unanesthetized rabbits are placed on their sides, and a 20-gauge short needle mounted on a 2 ml syringe is gently pushed through the intercostal tissues between the 5th and 6th ribs. After some experience, injuries to the heart and puncture of other tissues were eliminated, and the inoculum of 1 ml was readily placed in the center of the lower lobe. The results of titration to determine a desirable challenge are given in Table 2. Even old breeding rabbits could be fatally infected with as few as 100 virulent organisms. Challenge doses of 110 to 96,000 P. pestis 195/P regularly killed rabbits 8 months to 1 year old.

TABLE 2

TITRATIONS OF LETHAL DOSES OF P. *pestis* 195/P GIVEN TO RABBITS BY INTERCOSTAL INJECTION.

Number of P. *pestis*	Results Survivors/Total	Average Day of Death
96,000	0/5	4.0
48,000	0/5	4.5
24,000	0/5	4.8
12,000	0/5	5.2
6,000	0/5	6.0
1,760	1/5*	5.6
440	0/5	5.0
110	0/5	5.6

*The surviving rabbit had dislodged the needle, and the injection was made partially into the pleural cavity and subcutaneous tissue. This infection was not fatal.

Weight of Rabbits: 3-4 kg

Within 24 hours, the rabbits were febrile (temperature 40-41°C). For the next 2 or 3 days, sharp daily remissions and isolation of P. pestis from the circulating blood foretold the developing septicemia: first, labored respiration and anorexia, then weakness and ruffling of the fur, and finally sudden collapse.

At autopsy, the nares of some animals contained blood-tinged foam. The subcutaneous capillaries -- in fact, all the vessels of the serous membranes -- were injected. Blood-tinged exudate was present in one or both pleural cavities, and fibrin covered the pleurae of the inoculation site and adjacent lobes of the lung. In some animals, the pericardial surface carried strands of fibrin. The lobes of the lung were heavy and filled with edema. Small patches of hemorrhagic consolidation at the site of injection were difficult to distinguish from the universal edema. Only microscopic examination revealed areas of lobular and lobar pneumonia with extensive hemorrhagic exudation and emboli containing plague bacilli. The trachea was filled with blood-tinged foam, and the pattern of dark hemorrhagic intercartilaginous spaces was typical of Pasteurella infections in rabbits. The spleen and liver were slightly enlarged, and there were signs of developing nephrosis. Pasteurella pestis was demonstrable in large numbers in blood smears and cultures.

The autopsy findings of plague septicemia with lung edema, fibrinous pleurisy, and but little pulmonary consolidation re-emphasize the fact that septicemic plague, not pneumonic plague, results when P. pestis is introduced into the respiratory tracts of rabbits.

In small pilot experiments, the recovery of immunized rabbits from plague induced by intravenous or intraperitoneal challenge with virulent plague bacilli was followed 10-30 days after challenge by CF titers ranging from 1:64 to 1:256, bacterial agglutinins of 1:64 to 1:512, hemagglutinins of 1:8192 to 1:16,384, and MPI below 1. Frequently the MPI in this series indicated that even diluted (1:2) serum would protect mice against 100 MLD of a virulent P. pestis strain. Resistance was reflected by the antibody response measured in these tests.

The immunity response provoked by antigens and vaccines differed from rabbit to rabbit, but antibody levels after immunization did not reach the heights observed after challenge in the foregoing experiment. The tests included the CF and hemagglutination determinations with Fraction I and with toxin; bacterial agglutination; and MPI. The appearance, the level, and the persistence of antibodies also varied according to the suspending medium -- water or oil. By the 14th day after 3 inoculations of aqueous solutions, the antibodies had reached their highest levels. Decline by the 30th day was marked, and by the 90th day,antibodies had almost disappeared. After booster inoculations on the 90th day, levels were negligibly higher than those reached 2 weeks after the basic immunization series. The antibody response was quite different, however, when the antigen was incorporated in adjuvant. The peak was reached on about the 30th day, and in some animals was still maintained on the 90th day. The persistence of antibodies and the response to re-inoculation with the same or similar antigen are significant. In general, with adjuvant the antibody levels rose more slowly and attained higher levels, reaching their peaks after the booster. The water-in-oil emulsion adjuvants prolong the stimulation of an antibody-producing mechanism in a larger number of cells. The protracted stimulus results in proliferation of the antibody-forming mechanism, in respect to both cells and intracellular units, and the response is proportional to the original number of mesenchymal elements affected by the initial stimulus (White 1967).

In an experiment comparing two different _P. pestis_ isolates in aqueous form or in adjuvants (American "Yreka" and Indian 195/P agar-grown formalin-killed suspensions), rabbits were each given 1.4 mg antigen per ml solution in either a single dose or in two doses intramuscularly. The antibody response was measured on the 35th and 56th days. The only interesting difference discernible was the more vigorous antibody response (HA up to 1:16,384; MPI, 12 to oo) with the _P. pestis_ 195/P vaccine when it was suspended in adjuvant. Despite high antibody titers, the aqueous preparation in two doses protected only 5/8, while the adjuvant vaccines conferred immunity upon 9/10 rabbits; 10 controls succumbed on the 5th day to intercostal pulmonary infection. At autopsy, there was evidence of specific immunity in the 4 rabbits which were not completely protected. The inoculated plague bacilli appeared to be localized in a small pneumonic area, with relatively few viable _P. pestis_. Growth and invasion had been suppressed, since the bloodstream was sterile. This type of localized pneumonia has been observed only in rabbits treated with vaccines. It is particularly noteworthy that the mouse protection indices were normal (15-16) at the time of challenge. Two doses of purified Fraction I in saline or a single dose in adjuvant stimulated only _in vitro_ serum antibodies. However, both _in vitro_ and _in vivo_ antibodies were superior in the animals inoculated with adjuvant preparations. Resistance to pulmonary infection was incomplete; all the challenged animals were ill with fever, anorexia, and ruffled coats; 2 inoculated with aqueous Fraction I died (days 3 and 7), with lesions of local pneumonia and toxemia.

The antigenicity of live attenuated plague vaccine strains may be gauged in rabbits inoculated subcutaneously with dilutions of reconstituted freeze-dried vaccine. Antibody levels were determined on the 6th, 12th, 20th and 30th day. The E.V. 76 Saigon, even in the standard dose of 420 million viable cells, merely induced the appearance of HA, but effected no changes in the MPI. In contrast, E.V. 76 Paris, the standard vaccine strain used by Girard, 180 viable P. pestis had already reduced the MPI to 2, and, correspondingly, HA titers reached 256 on the 12th and 512 on the 30th day. At least 20 million E.V. 76 Saigon were required to stimulate such HA titers, which were not paralleled by MPI below 5. The nature of these differences in antigenicity has not yet been determined. In this connection, it is recalled that the subcutaneous inoculation of living P. pestis E.V. Saigon in the dosage of 420 million viable organisms induced no changes in the MPI of human subjects. It will be necessary to study systematically the antigenic activity of live attenuated P. pestis on rabbits with special reference to protective antibodies.

V. The Role of in vitro and in vivo Laboratory Assays in Determining the Protective Efficacy of Killed and Live Plague Vaccines.

From the somewhat unmanageable array of facts presented in the preceding paragraphs, strong support has accrued to the observations of Jawetz and Meyer (1944) that the in vivo passive mouse protection tests permit fairly accurate and reproducible titrations of anti-infectious and antitoxic antibodies, not merely in sera from hyperimmune antiplague rabbits but in the sera of other animals as well. The sera of convalescent human patients, nonhuman primates, guinea pigs and mice recovered from plague have yielded mouse protective indices below 5. Further systematic immunization studies on guinea pigs and mice, and more recently on the highly susceptible nonhuman primates -- langurs (Presbytis entellus) and African vervets (Cercopithecus aethiops) -- demonstrated that in these vertebrates, recovery from plague is ensured by MPI below 5. Whenever the MPI was below 5, the animal was able to resist massive subcutaneous infection without local or systemic reactions. The observations in langurs and vervets are particularly convincing. However, the essential question of what mouse protection index level measured in the peripheral blood of man reflects a relative state of immunity against plague, remains unanswerable. Available evidence suggests that humoral antibodies mediate the important cellular phase in the immunity mechanism. In some still unknown manner, the immune body fluids exert their most important influence by altering P. pestis organisms so as to make them more accessible to intracellular enzymes and thus prepare the bacteria for destruction (Meyer 1950). Experiments with langurs have conclusively shown that vaccines or antigens, with or without synergists or adjuvants, in dosages and inoculation schedules which fail to stimulate significant mouse protective antibodies, are usually nonprotective against experimental infections.

Therefore it is confidently reasoned that in man the same mechanism is operative. Only antigenically complete vaccines capable of eliciting low protective indices in approximately 60 percent of not less than 5 langurs deserve clinical trials on man.

The mouse protection test require at least 5.5 ml of serum, a uniformly susceptible strain of mice, a stable virulent strain of *P. pestis*, and skillful technicians to perform the intravenous inoculation of the serum, followed immediately by the standardized 100 LD_{50} infection dose. Clinical trials on inmates of well-equipped institutions together with constant supervision by physicians, have provided adequate serum specimens essential for studies on the immunization of man against plague. (Under field conditions, these requirements are rarely fulfilled.)

In carefully conducted tests, measurable *in vitro* antibodies against the Fraction I antigen -- i.e., hemagglutinins and complement fixing antibodies -- generally reveal a fairly close relationship with mouse protection indices. For example, sera derived from langurs or vervets vaccinated with killed or live vaccines yielded the following correlation values: MPI, oo to 3; hemagglutination titers, 1:2048 to 1:32,768. With MPI above 10, the hemagglutination titers were between 64 to 512 when the sera were tested on the 28th day after vaccination. Hemagglutinins appear first on the 6th day and rise rapidly, while the protective antibodies appear later and more gradually, particularly with large doses of live vaccine. Such values in individual animals may be variable, and in 5-10 percent correlation of the 2 types of antibodies may not be apparent (Reports 17 and 20). Over a period of 3 to 6 months, the MP index gradually rises and in many individual monkeys may return to normal levels. Hemagglutinins likewise decline slightly, but, at least over the longest observation period of 1 1/2 years, disappear entirely. Similar correlations have been recorded and described for human subjects in the course of clinical trials with plague vaccines as well as in the case of patients recovered from plague.

Additional significant findings warrant comment: In the course of vaccination experiments with nonhuman primates, it was noted that killed vaccines without synergists or adjuvants *never* stimulated complement fixing antibodies, whereas recovery from infection and the inoculation of live vaccines invariably induced titers as high as 1:512. The high titers gradually declined over a period of 6 to 8 months at approximately the same rate as the MPI. The studies by Payne and his associates (1956) with sera collected in Madagascar fully confirm results of the experimental studies on monkeys -- i.e., hemagglutinins in appreciable amounts persist for months and even years after recovery. More important, however, was the detection of antibodies against Fraction I and toxins of P. pestis in the sera of a few Malagasy with no known history of plague or vaccination. These observations have since been confirmed by Colonels John D. Marshall, Jr., and Dan C. Cavanaugh (personal communication) in Vietnam and Dr. M. Isaäcson in South Africa, and raise the possibility that unrecognized and possibly clinically inapparent infections with P. pestis do occur in persons residing in areas endemic for plague. In Vietnam, a group of 37 vaccinated men had significant hemagglutinin titers when they began to collect rodents and fleas; within 2 months, 12 revealed definite rises in these antibodies. More striking and even more suggestive were the findings in a group of 122 men who had repeatedly received vaccine inoculations: 44 had no hemagglutinins when they entered field service in heavily infected territories. When this group was examined 6 months later, only 4 had no serum antibodies, while 118 yielded titers of 1:64 to 1:4096. Here an unorthodox and purely speculative theory that unrecognized small-dose infections derived from bites of infected fleas might have been responsible for such antibody responses deserves consideration. At a recent meeting of the Commission on Immunization, Colonel John D. Marshall, Jr. reported that 98 percent of a group of individuals given 6 or more immunizations had detectable antibodies; in fact 28 had detectable complement-fixing antibodies. All the CF positive persons had histories of contact with P. pestis. Another group of 6 individuals, who had never been exposed to cultures of P. pestis and who had both HA and CF antibodies, all related histories of actively (10 or more days per year) engaging in hunting or dressing game barehanded in a known epizootic plague area.

In view of these recent observations on humoral antibodies, it is imperative that, aside from measurements of the protective and antitoxic properties of plague vaccines, the hemagglutination and complement fixation tests be included in clinical trials. The evidence suggests that in contemplated field trials, the in vitro serologic techniques requiring smaller amounts of serum may serve as substitute tests.

VI. Principles Relative to the Immunization of Man Against Plague.

Experience has taught that nonfatal clinical infections commonly protect against a second attack, or at least transform re-infection into a milder form of disease. Persons with a history of plague, such as nurses or hospital attendants, were, according to custom, employed in isolation wards, because one attack of plague was believed to confer lifetime immunity. Critical survey of the literature and analysis of records reveal that one bout with plague does not induce permanent immunity; many re-infections have been bacteriologically confirmed (Girard 1963).

Development of immunoprophylaxis against plague commenced soon after the discovery of the causative agent of the disease. The first type of vaccine for active human immunization was devised by Yersin, Calmette and Borrel of the Pasteur Institute (1895) and the German Plague Commission (1899) (Kolle, Hetsch and Otto 1904); it was prepared from 48-hour fully virulent agar cultures killed by heating at 58°C or 65°C for 1 hour; 0.5 percent phenol was added as a preservative. Infection with small doses of virulent cultures produced some protection in rabbits, rats, guinea pigs and even monkeys (Wyssokowitz and Zabolotny, 1897) after repeated inoculations of the killed bacillary suspensions. About a year later Haffkine in India (1897) obtained similar results with broth cultures of freshly isolated, highly virulent *P. pestis* kept in the dark from 6 to 8 weeks at room temperature (23-29°C) and sterilized by 15 minutes in a water-bath at an average temperature of 55°C. After 0.5 percent phenol had been added and sterility tested, this so-called "whole vaccine," without being subjected to any particular potency tests, was used in dosages of 3-4 ml in the first vaccination of man against plague. A small-scale study was conducted with 346 prisoners of Byculla Jail in Bombay in 1897. Haffkine inoculated about half the group at the beginning of a 7-day outbreak and left the other half uninoculated. During this period, 2 cases (non-fatal) among 147 inoculated and 12 cases (6 fatalities) among 172 uninoculated individuals resulted. In a second study of plague vaccine, performed in Umarkadi Jail in Bombay, Haffkine inoculated "one individual among two"; within a 30-day observation period, 10 cases of plague infection with 6 deaths occurred among 127 uninoculated prisoners and 3 cases with no deaths among 147 inoculated men. In a well-planned study in Undhera villages, he recorded 27 cases and 26 deaths among 64 uninoculated and 8 cases and 3 deaths among the inoculated. These trials were not "controlled studies" in the present sense, but they did serve to indicate the effectiveness of the Haffkine killed *P. pestis* broth culture, although the exact degree of efficacy is difficult to assess. Despite the total evidence favoring Haffkine's vaccine, the Indian Plague Commission (in 1899 and 1910) was nevertheless critical of its value.

Investigation of the epidemiologic effectiveness of the vaccines revealed a difficulty in determining the decline in morbidity among both the vaccinated and nonvaccinated; studies on the reduction in mortality were therefore initiated. Taylor (1933) analyzed the records concerning the effectiveness of the killed Haffkine vaccine in India and found that among 3,227,842 nonvaccinated persons exposed, 56,668 cases of plague with 50,735 deaths (85.1 percent) occurred, while among 1,937,213 vaccinated, there were 4,128 cases with 1,620 deaths (39.3 percent). Many authors (Girard and Robic 1936; Zhukov-Verezhnikov 1940; and others) criticized this revelation of decline in plague mortality, due to vaccination, as exaggerated. Even in India, analyzing data from a plague emergency hospital, Simeons and Chhartre (1946) found that the comparative mortality rate among vaccinated and nonvaccinated patients differed only by 2.62 percent (16.25 percent versus 18.67 percent); thus they concluded that the value of a single inoculation of 2 ml killed vaccine "as a method of reducing the mortality is rather dubious." Their paper in the *Indian Medical Journal* strongly supported conclusions already published in 1933 by the Office International d'Hygiene Publique seriously questioning the value of the Haffkine vaccine as a plague prophylactic and encouraging the study of live plague vaccine. In fact, reviewers of plague vaccine studies on man (Petrie 1929; Taylor 1933) stated that research was needed to discover a plague vaccine that would confer the maximum degree of immunity with the minimum amount of discomfort to the recipient.

Disappointed with the doubtful efficacy of dead vaccines, Girard and Robic (1932 and 1936) and Otten (1936) recommended the use of live vaccine, prepared from attenuated plague strains. After its high effectiveness and safety had been determined in experiments on animals and human volunteers, the fluid vaccine was then given subcutaneously in field trials in single injections in a dosage of 1 to 3 billion bacilli for adults and about half that amount for children. Reactions to the inoculation were reported as moderate. In Java, the attenuated strain -- known as the Tjiwidej (TJW) and obtained from rats -- was used for mass inoculation of 37,500 persons during an outbreak. At the end of the epidemic, the mortality was 14.6 percent among the vaccinated and 85.4 percent among the nonvaccinated. In the dosages used, the vaccine was not protective against pneumonic plague. Since Otten believed that immunity lasted for 5 months, he recommended revaccination after this interval. In Madagascar, Girard and Robic (1938) vaccinated some 47,000 persons, comparing them with a group of 60,000 nonvaccinated. During 5 months of observation, 22 cases of plague occurred among the vaccinated and 100 in the control group, indicating that about two-thirds of those receiving the live vaccine had acquired protection. Mild in the Malagasy, local and systemic reactions after the inoculations were more severe in Europeans than the reactions which followed the administration of typhoid vaccines (Rotman 1945).

At the 1965 WHO seminar on plague control in the USSR, Dr. B. Cvjetanovic, Chief Medical Officer of the Bacterial Diseases

Section of WHO, in a report on "Earlier Studies of the Effectiveness of Plague Vaccines in Man," delivered this significant statement: "Except for a few studies by Haffkine (1906) and Otten (1936), no other studies of the effectiveness of plague vaccine in man have been undertaken which deserve to be called 'controlled' studies (as defined by Hill 1951, and Pollock and associates 1965). The information available from these studies, in spite of some reservations, indicate that dead plague vaccine is to some degree effective and that live vaccine is perhaps superior. However, its exact degree of protection and duration are unknown." He also quoted R. Pollitzer's observation (1954) that "The statistical evidence presented in regard to the efficacy of plague vaccination, though ample, is often not satisfactory, mainly because the groups of the vaccinated and unvaccinated were not under the same risk of infection."

During the discussion, Professor N. I. Nikolaev of the All-Union Scientific Research Institute on Plague Control "Microbe" at Saratov (USSR) stressed that in past epidemics, vaccination with living attenuated vaccines, as practiced by Otten (1941), Girard (1963), Grasset (1946), Devignat (1949), and Osolinker (1960) (reviewed by Girard 1963), notably reduced both the infection rate and the mortality of vaccinated subjects. After reviewing the excellent and well-planned experimental studies of Professor E. I. Korobkova and her associates (1956, 1962), he added his own observations in 1945 in Inner Mongolia, where a *dry* live vaccine of the E.V. strain was used subcutaneously in the dosage of 1.5 billion bacilli.

The plague mortality among 19,889 *vaccinated* persons was 2.5 percent while among 4,520 *nonvaccinated*, it was *288 per 10,000*, which is indeed significant. He then referred to a recent field trial reported by V. P. Smirnov (1962) on the epidemiologic effectiveness of dry live E.V. vaccine used in the Mongolian People's Republic and administered subcutaneously (1.5 billion bacilli), via the conjunctival route (1.5 billion), or by the combined subcutaneous and conjunctival routes (each 1.5 billion). Of 7,225 persons vaccinated subcutaneously, 6 individuals (or 8 per 10,000), compared to 33 of 3,331 (or 100 per 10,000) nonvaccinated, contracted pneumonic plague. Of 115,035 persons vaccinated via the conjunctivae, 2 contracted plague (1 pneumonic and 1 bubonic). Of 31,278 nonvaccinated persons, 69 contracted plague, including 48 cases of fatal pneumonic plague. In other plague foci, 2 of 104,931 persons vaccinated according to the combined method contracted plague and 1 recovered; out of 39,350 nonvaccinated persons, 26 fell ill (16, fatal pneumonic; 9, fatal bubonic, intestinal; 1 recovered). Conjunctival vaccination was chosen for 2 reasons: (a) to protect the individuals against respiratory infections, and (b) to reduce the discomfort of vaccination (although Professor Nikolaev added that the conjunctival vaccination produced mild conjunctivitis lasting 2 days). Finally he reminded participants in the Seminar that the difficult evaluation of the epidemiologic effectiveness of human vaccination against plague is further complicated by the epizootic background of plague epidemics; the severity and fatality of the disease in different natural foci are not always taken into consideration in evaluation of the observed results. Professor Nikolaev's entire presentation reflected the conclusions of Soviet plague immunologists that, both experimentally and in epidemiologic trials, the low effectiveness of dead vaccines and the high effectiveness of live attenuated vaccines, particularly E.V., have been demonstrated. In personal interviews, Drs. I. V. Pechnikova and V. G. Philipenko of the Vaccine Division, Stavropol Branch of the All-Union Scientific Research Institute on Plague Control "Microbe," disclosed that the standardized, dry antiplague vaccine, grown either in fluid or an agar medium, is suspended in 10 percent saccharose solution; a 1.5 percent solution of gelatin serves as the medium for desiccation. The viability of the dried product, containing less than 5 percent moisture, is variable, but a preparation of 35-40 percent living organisms is considered excellent. Batches with less than 20 percent viability discarded.

Conversations with Professor E. I. Korobkova revealed that vaccine strains, even in the lyophilized state, may lose their immunogenicity. Repeated passages in animals, particularly in the presence of nontoxic doses of iron salts, are required to restore and even enhance the immunogenic properties. From the results of her extensive studies, Professor Korobkova believes that all vaccine strains must immunize guinea pigs, that they must contain the antigens FI and VW, and that they must be dependent upon uracil for growth but must not produce pigment (i.e., must be P negative) on media containing haemin. These determinants are typical for the E.V. strain, by far the most immunogenic vaccine strain known to USSR scientists. In her experiments, Professor Korobkova combined subcutaneous, cutaneous and aerosol vaccination, and produced a high level of immunity to aerosol infection. Furthermore, she found that large doses of vaccine were necessary to ensure a relatively long non-infectious phase of immunity-persistence, with inoculated bacilli in the tissues for more than 3 weeks, followed by the sterile phase. The most important role in the protection of the animal host or man is that of the "immunologically competent cells" which react with the antigens in the first stage of immunity development and later serve as the source of other cells capable of synthesizing antibodies or resisting toxins. The former cells possess "immunologic memory"; having received antigenic stimulation, they give an "immune response" after repeated administration of the antigen. When asked how these responses were measured, Soviet scientists replied that they do not consider serological evidence of the development of antibodies as a possible indication of immunity. They are unfamiliar with the passive mouse protection test. Moreover, they insist that they have never been able to demonstrate antibodies 15 to 20 days after single doses of vaccine in guinea pigs proven immune to plague. Hence they view with considerable skepticism deductions on the immunogenicity of vaccines for man based on his serologic response. On the other hand, at the Institute at Saratov, the immune responses of individuals to dried live vaccine have been tested with the aid of the allergen "pestin," of unknown composition and dosage. A positive intracutaneous allergic reaction of the delayed type is regarded as indicative of immunity to plague and is claimed to be useful in determining the time for revaccination. It was repeatedly stated that revaccination is advisable because the immunity definitely declines by the 6th month. The fact that pestin may cause systemic reactions accompanying the pustule formation, with extensive hyperemia lasting for several days, was not reported in the paper read but was explained in the subsequent discussion. At least 18 percent of the reacting subjects had malaise, headache and pyrexia. Proof that the so-called "immune response" in man, evidenced by hypersensitivity, is associated with resistance to plague was not forthcoming. It is regrettable that in the course of large-scale prophylactic inoculations of Soviet field and laboratory personnel, serologic examinations were not performed to verify studies made in Madagascar (McCrumb *et al*. 1955; Payne *et al*. 1956). Such studies are particularly urgent because, in the absence of controlled field trials during plague epidemics, promising laboratory serologic methods capable of measuring the efficacy of vaccines in animals are now available.

To date, while awaiting the rechecking of such methods by other workers, the principal investigator hesitates to declare that the immunogenicity of vaccines for man can be adequately appraised with the aid of these newer procedures. Despite extensive data collected in the course of surveys and limited immunization studies on man using dead vaccines, final conclusions about the merits of serologic tests should, for the present, be formed only with reservations.

All the evidence presented by Soviet scientists and public health workers during the 1965 Seminar supported the conclusion that the live dried E.V. plague vaccine is probably a highly effective prophylactic for inducing immunity in man.

VII. The Present Status of Killed Plague Vaccines.

In response to the presentations of the Soviet immunologists, the principal investigator reviewed his experiences with killed plague vaccines.

On October 22, 1941, the Subcommittee on Tropical Diseases, National Research Council Committee on Medical Research (Meyer and McCoy 1964), passed the following resolution: "Resolved that, even though the available knowledge does not seem to afford definite evidence of the benefits from the use of plague vaccines, it is advisable to vaccinate with killed plague bacilli of an approved strain all military or naval personnel under serious threat of exposure to bubonic plague."

In March, 1942, under the guidance of the writer, a commercial biological laboratory was instructed to proceed with the preparation of a formalin-killed, agar-grown suspension of virulent plague bacilli in saline and to determine (at the G. W. Hooper Foundation for Medical Research, University of California) the relative protective potency of the vaccine for mice, according to the method of Sokhey and Maurice (1937 and 1939). Results of studies involving the manufacture of the killed vaccine, and experiments dealing with various antigenic fractions and their protective potency as measured on mice and guinea pigs, have been reviewed (Meyer _et_ _al_. 1948; Meyer 1953).

In search of an effective prophylactic inoculation against plague, the majority of workers adhered to the principle that a preparation or antigenic fraction which protects rats, mice and guinea pigs against experimental plague represents good presumptive evidence of efficacy in man. The standard procedures developed by researchers since 1942 and adopted to evaluate killed plague vaccines included assays on mice as well as on guinea pigs. It was found that mice inoculated in 2 steps with doses of 0.002 ml USP plague vaccine resisted challenge infection with 2000-4000 virulent plague bacilli, but the prophylactic value was relatively low for guinea pigs. When administered in the dosage customary for human immunization (1.5 mg = 3 x 10^9 organisms), only 10-20 percent of the guinea pigs survived challenge infection.

Other experiments provided convincing evidence that the degree of guinea pig protection is increased with large, repeated doses of antigens or with small doses precipitated with a synergist (aluminum potassium sulfate (alum) or aluminum hydroxide, but not calcium phosphate). In some tests, 80-100 percent of the guinea pigs were protected with antigens which in the unprecipitated state conferred resistance upon only 10 percent of the animals.

It was further observed that differences in the efficacy of killed vaccines are complicated by the great heterogeneity in the susceptibility of this animal species widely used in laboratory experiments. In suspension made with virulent or attenuated plague isolates, it is the actual mass of aggregate bacterial protein rather than the method of killing (treatment with formaldehyde, acetone at -70°C, pure ethanol, potassium sulfate, glycerol, irradiation, etc.) which controls the immunogenic effect on guinea pigs.

Employing vaccine dilutions emulsified in an adjuvant, a so-called "guinea pig potency test" similar to the mouse potency test has been included in the evaluation of killed vaccines. Altogether, 20 USP vaccine lots tested on the inbred Hartley strain of guinea pig protected 60-70 percent infected with an LD_{50} of 200,000 virulent P. pestis. The results of these tests, which irrefutably prove that guinea pigs can be protected against plague with killed vaccines, have recently been confirmed by other workers (Smith and Packmann 1966). A nontoxic filtrable vaccine, developed from the attenuated E.V. 76 P. pestis strain, protected approximately 69 percent of several hundred guinea pigs against virulent subcutaneous infection. These animals had each been inoculated with 2.0 ml of the preparation in 2-dose schedules. Under identical conditions, the USP plague vaccine conferred immunity upon 43 percent of the vaccinated guinea pigs. Compared with the USP vaccine, the filtered vaccine owes its superior immunogenic efficacy to a greater content of antigen and to substances of high molecular weight.

There is no need for lengthy discussion of the puzzling paradox observed during early studies with the soluble protein Fraction I plague antigen, which protected mice but not guinea pigs. This so-called "immuno-unresponsiveness" (or paralysis) is attributable to a prolonged surface blockade of the membranes of the reticulo-endothelial cells by large doses (more than 50 μg) of Fraction I. In comparison with guinea pigs, mice catabolize the protein faster, eliminate Fraction I sooner, and do not become immuno-unresponsive. Very small doses of Fraction I (5 μg) in adjuvant protect 100 percent of guinea pigs against severe plague infection (Spivak et al. 1958; Walker 1962). In one experiment, inoculations with 2 mg aqueous Fraction I followed by 0.5 mg in adjuvant solidly protected only 2 of 6 langurs.

Potency tests of killed vaccines on macaques generally proved unreliable because of the relatively low and variable susceptibility of this species. Clinical trials on human subjects before 1944 established the safety and dosage of the vaccines which could be tolerated with the least amount of discomfort in terms of local and systemic reactions. Methods were then developed to measure indirectly, by serum tests, the responses of various animals, including man, to plague antigens. The passive mouse protection test, and later the passive hemagglutination test, provided evidence that killed plague vaccines with proved immunogenic potency for mice and guinea pigs stimulate the appearance of these antibodies in approximately 50-60 percent of human subjects.

At the end of World War II, the final report on studies to develop a standardized type of killed plague prophylactic (Meyer and McCoy 1964) ended with the cautious statement: "Although the formalin-killed virulent P. pestis suspension proven effective in tests of animals has not yet had extensive field trials, there is no reason to doubt that it enhances the immunity mechanism in the body sufficiently to warrant its use. Inoculation with killed plague vaccines or antigens offers a practical approach to lowering the attack rate in endemic areas." All available official and nonofficial reports were carefully searched for evidence to support the validity of this statement. The fact that no one in the US Armed Forces contracted plague, despite potential exposure in the Mediterranean area and the Orient, attested to the efficiency of the cooperative measures taken by the Medical Department of the US Army and civilian public health departments. However, in the British Middle East Force, there were 26 cases of bubonic plague, including 5 deaths (19.2 percent), involving 12 Indians, 6 East Africans, 5 British, 1 Italian, 1 "Cape-colored" and 1 European Jew.

The efficacy of the formalin-killed plague vaccine could not be determined under field conditions, since the insecticide (DDT) effective against the vector, along with certain microbiological agents useful for treatment, and, epidemiologically more significant, for prophylaxis, had been employed simultaneously. However, it is reported that prophylactics prepared in India and China and used in field trials had lowered the attack and mortality rates, albeit not with overwhelming success. The preparations afforded inoculated individuals a better chance of recovery when they were treated with sulfonamides or antibiotics or both, preferably early in the course of the disease. Reports on clinical reactions noted in the course of inoculation were important to the development of effective prophylactics. Local and systemic reactions resulting in the temporary incapacity to perform their duties were noted in vaccinated persons following the second inoculation (1-2 ml) of the killed vaccine.

The health authorities of one Pacific Island were forced to discontinue the use of the Haffkine vaccine imported from India because of the severity of vaccination reactions in the civil population. Subsequently, more than 4600 persons accepted vaccination with the Army preparation without complaint.

The antibodies active in the passive mouse protection test have not all been identified. The immunoelectrophoretic analytic procedure (IEAP), detailed in Report 14, 1964, and McNeill and Meyer (1965) revealed that generally 2 or more precipitin lines were demonstrable in the sera from patients convalescent from plague and individuals inoculated and properly boostered with vaccines. The most frequent lines were identified as Fraction I and murine toxin antibodies. The third line was attributed to the antibody against antigen E (Lawton 1960). No attempt was made to identify other lines.

Ordinarily sera from recovered plague patients had complement fixation and high passive hemagglutination titers. In view of the extensive studies on experimental rodent and primate plague during the past 20 years, it was anticipated that FI, the highly immunogenic component of the *P. pestis* antigenic complex, would likewise induce the dominant and persisting antibody in naturally occurring human plague. The demonstration of precipitin lines identified as murine toxin was not surprising, since (1) observations on rodents and primates with the passive hemagglutination technique had established the high antigenicity of the toxin, (2) the sera of unvaccinated Malagasy recovered from human plague revealed specific titers of 1:20 to 1:10,240 in HA tests with murine type of toxin (Payne *et al*. 1956), (3) the original research on the immunizing substance of the plague bacillus by Rowland (1910) (summarized by Petrie, 1929) stressed that to be efficient a vaccine must initially have a toxic action, and (4) in the past, no effort was spared to produce a plague antitoxin, although it had been clearly demonstrated by Markl (1901) that the serum is feebly or not at all antitoxic. The appearance of toxin precipitin lines demonstrable by the IEAP indicates that in the sera of plague patients the antigenic toxin is part of the living plague organism *in vivo*.

A comparison of the antibody patterns of plague patients and those of subjects inoculated with plague antigens revealed interesting correlations (Report 15,1965). The geometric mean titer in the plague patients was 1:8 for complement fixing antibodies and 1:2048 for hemagglutinins, in striking contrast to mean titers of only 1:2 and 1:128, respectively, in inoculated subjects. On the other hand, the number and combination of precipitin lines demonstrable by the IEAP were similar in both groups of sera.

In the course of a plague infection treated with streptomycin, the larger numbers of disintegrating organisms release more FI antigen and exert a stronger stimulus than does the fixed smaller amount in the vaccine. This may partly explain the observation that 16 of the sera from 17 plague patients had FI lines, while only 31 (43 percent) of the sera from 72 vaccinated individuals demonstrated FI precipitin lines.

The lower percentage (31 percent) of toxin lines in the sera of the vaccinated in contrast to that of the plague-recovered patients (53 percent) may be attributed to the nature of the plague vaccine inoculated. The formalin used to kill the virulent plague bacilli converts part of the toxin to toxoid. Of primary importance is the discovery that the serum-precipitin pattern of persons inoculated with formalin-killed plague antigens mirrored that of patients 6 to 35 days after recovery from plague. This type of replication is noted only following booster-dose inoculations of persons receiving 2 basic inoculations totaling not less than 2.5 x 10^9 killed plague bacilli 1 month apart. Regardless of the similarity of the reactions, the antibody response expressed in passive hemagglutination titers in vaccinated subjects does not attain the levels of that reached in patients convalescing from plague.

Extensive tests on laboratory mice, guinea pigs, rabbits and primates (detailed in Reports 11, 12 and 13) have repeatedly demonstrated that serologic response and degree of immunity to experimental infection are superior when the antigenic mass (the number of dead plague bacilli or mg total N/ml) is relatively large (4.0 to 8.0 x 10^9), but local reactions in the form of induration, lymph node swelling, and tenderness of the inoculated muscles are frequent and severe. The inoculation of guinea pigs with 5,260 million dead plague bacilli in 2 equal doses 1 month apart induced the appearance of peripheral antibodies in significant titers (CF, up to 1:16; HA, 1:8192; average MPI, 1 to 3.7) and resistance to intranasal challenge,fatal to controls in 3.8 days, 23 days after the last inoculation. Somewhat limited experience suggested that man would react as severely as guinea pigs. For example, in 1950, a vaccine preparation consisting of 1.5 billion acetone-killed avirulent strain A1122 and E.V. 76 in oil-water-aluminium stearate adjuvant, administered intramuscularly to guinea pigs, resulted in swelling and lameness of the inoculated legs. But since the preparation fulfilled Rowland's specification that an efficient vaccine must originally have some "toxic" action, protected guinea pigs against severe challenge, and induced peripheral protective (average MPI, 4.5) and complement fixing (up to 1:16) antibodies, it was decided to try this vaccine on human subjects. The consequences were serious: in 8 of 21 men inoculated, severe local reactions were followed by abscess formation, requiring surgical drainage in 3 (details in Report 1, 1951). As anticipated, the antibody response was disappointing due to the fact that single dose inoculations had deliberately been chosen. In view of this experience, subsequent human immunization trials avoided large doses of antigen. Even the atoxic Fraction I was apt to sensitize the subjects and cause local and systemic reactions upon repeated inoculations. Despite the foregoing experiences, however, attempting to improve the immunity response to alum-coated plague vaccine was irresistable. Thus in a series of vaccine titrations to determine the optimal concentration causing no serious side effects, a total concentration of 8.0 x 10^9 in 2 doses of 4 x 10^9 was tested on human subjects (Report 12, p. 21). This concentration of vaccine caused local reactions in 66 percent and systemic reactions with fever in 40 percent of the subjects. Reactions were particularly marked when the same concentration was administered as a booster dose 6 months after basic immunization. Army personnel who observed and studied the reactions considered the side effects of this vaccine dose to be incompatible with the prevailing standards for routine mass vaccination.

After 1945, laboratory research on plague vaccines was continued in the form of analyzing the techniques of serological methods: In particular, the following tests were improved and evaluated: (1) the *in vivo* mouse protection test, (2) the *in vitro* passive hemagglutination test, (3) the complement fixation test with Fraction I, (4) the macroscopic agglutination test, (5) the opsonocytophagic test, and (6) the complement fixation test with murine toxin.

(A). Formalin-Killed Virulent P. pestis Saline Suspension Vaccines.

The killed plague vaccine currently in use in the USA has been studied by means of standardized procedures, with results best illustrated by the following investigation: A new agar culture medium (E), which contains less than 0.003 ml of blood groups Anti-A and B substances, was developed in collaboration with Cutter Laboratories (Reports 16 and 17). The plague vaccine, known as USP, consists of an aqueous suspension of virulent *P. pestis* (Indian isolate 195/P) grown on the agar medium in Roux bottles at 37°C for 48 hours. The organisms are harvested in physiologic saline and killed by adding formol to an overall concentration of 0.5 percent. The standardized suspension is supplied in 20 ml vaccine vials, each containing 2 x 10 bacilli (average total nitrogen, 0.167 mg per 1 x 10^{-9} organisms in suspension). It has an average FI, expressed by a CF titer of 1:512 and a mouse potency of 5.8. The ratio between various lots of vaccine and the reference standard has varied from 4 to 10; shelf stability at ±4ºC is at least 2 years.

The contents of several lots of randomly chosen vials, inoculated intraperitoneally or subcutaneously into guinea pigs (inbred Hartley strain) in the human dosage (1 ml = 2 x 10^9 organisms), followed by single booster doses (0.2 ml = 400 million organisms) given intramuscularly 1-2 months later, protect 50-70 percent when the vaccinated animals are challenged with 40,000 to 500,000 virulent P. pestis.

In a group of 9 langurs inoculated by the intramuscular route, the USP vaccine produced no local or systemic reactions. The hemagglutination test performed with their blood sera on the 35th day after basic inoculation demonstrated hemagglutinating antibodies in titers varying from 1:16 to 1:128, no complement fixation titers, and mouse protective antibodies (average 11), with an MPI of 10 or below in 3 animals. The langurs were given booster doses of 0.2 ml (400 million) USP vaccine intramuscularly 86 days after the first inoculation. At the same time, 1 animal not previously vaccinated was inoculated with the booster dose.

Significant antibody titers after the booster indicated at the time of challenge infection that the immune response expressed in hemagglutinin titers was vigorous, with an MPI below 5 in 4 animals and a moderately good MPI in 5. The langur given only the 0.2 ml booster dose had no significant antibodies. On the 40th day after booster inoculation (125th day after primary inoculation), the vaccinated animals, together with 3 nonvaccinated langurs as controls, were each subcutaneously infected with 131,000 virulent P. pestis 195/P.

The controls succumbed to bubonic-septicemic plague within 5 to 6 days; 1 langur inoculated with the USP plague vaccine, although given a booster, failed to develop a significant MPI (only 18) and died at the same time as the controls. Altogether, 6 of 9 langurs, with MPI of 1, 3, 4, 5, 7, and 1 animal with complete protection (oo) prior to challenge, survived. In an animal with an index of 13, an axillary bubo developed and yielded the inoculated strain of P. pestis in undiminished virulence on the 57th day after challenge. Three vaccinated langurs with MPI of 13 (2) and 15 succumbed to septicemic-metastatic pulmonary plague on the 16th, 21st and 35th days after challenge. The protracted disease, seen for the first time in langurs, resembled "mitigated" or "relapsed" plague in relatively resistant rodents. With the exception of 1 animal, all langurs surviving plague exhibited either no cutaneous reactions or well-defined fleeting erythemas and slight local indurations at the sites of infection.

Here it is important to emphasize that resistance to plague was indirectly reflected by an MPI below 5. None of the langurs with an MPI above 10 survived except for the animal with the axillary bubo. The high-titer antibody response following recovery persisted for 2 months; the appearance of complement fixing antibodies following infection is particularly significant. The killed USP vaccine, administered according to directions adopted by the US Army and civilian personnel in respect to dosage and schedule, conferred solid protection upon approximately *60 percent of the langurs experimentally infected with plague* by the subcutaneous route. Almost identical correlations and immunogenic efficacy have been recorded for other lots or combined vaccine preparations containing 2×10^9 formol-killed virulent *P. pestis*.

A *clinical trial on 25 human subjects* with the same lot of USP vaccine, employing intramuscular injection in a 2-dose schedule, proved informative. Local and systemic reactions were all mild; lymph nodes were palpable in 2 men. Subjects with mild respiratory infections complained of myalgia and malaise, but by the 72nd hour the reactions had disappeared. Passive hemagglutinins in titers varying from 1:8 to 1:256 were noted on the 8th and 16th days in 16 of 25 men. By the 28th day, all but 1 of the subjects had reacted with hemagglutinin titers of 1:16 to 1:1024. Within 3 months the titers had declined to lower levels or had disappeared in 25 percent of those vaccinated. Single booster inoculations of 0.2 ml (400 million) USP vaccine restored hemagglutinin titers to original levels.

In view of the findings in langurs, passive MPI determined *in vivo* should be given special consideration. Indices below 10, and particularly below 5, are usually found in susceptible nonhuman primates which *resist* challenge infection. In the 25 human subjects, 16 percent of the MPI were below 10 by the 28th day, with only 4 percent below 5. *Thus a single inoculation of vaccine confers little or no active immunity*. A small booster inoculation brought the MPI to below 10 in 65 percent of the men and to below 5 in 26 percent. Clinical trials on human subjects with other killed vaccines in a 2-dose schedule have yielded similar MPI recordings, considered adequate to confer relative immunity upon approximately 65 percent of human subjects.

Perhaps by mere coincidence, survival figures for human subjects inoculated in field trials in Kenya (Smidt 1929) with a locally prepared Haffkine vaccine (2 ml) represented a 61.8-65 percent protection rate, corresponding to the survival figures for human subjects who acquired protective antibodies after 2 antigenic inoculations with the USP plague vaccine. Exceptions have been observed, however, in individuals inoculated 10-20 years before inclusion in recent clinical trials. Invariably hemagglutination titers of 1:16-1:65,000, with MPI as low as 1, or complete protection of all mice inoculated with the sera, were recorded 12-28 days after revaccination. Killed vaccines of the USP type definitely sensitize human subjects to the extent that small, repeated booster inoculations activate the immune mechanism.

How then can protection, indirectly measured by serum antibodies, be brought to a level of 90 percent with vaccines which are free from side effects? Experiments on guinea pigs with a killed vaccine suspension, adsorbed or precipitated with aluminum salts, improved the percentage of survivors and lowered the MPI in human subjects. So far, vaccine preparations with alum tend to deteriorate relatively fast; studies to discover the reasons for such deterioration are in progress. Although local reactions are only slightly more frequent, even with intramuscular inoculation, general systemic reactions are approximately twice as common as those resulting from the USP vaccine without synergists.

Killed bacterial vaccines in oil adjuvants are, justly, out of favor: they are highly efficacious in guinea pigs and nonhuman primates, but in 1 clinical trial on human subjects, a killed (acetone) vaccine emulsified in "Bristol" (peanut oil and aluminum monostearate) and demonstrated to be 100 percent protective against very severe infections in guinea pigs, caused severe local reactions in 8 of 21 men upon intramuscular inoculation, followed by abscess formation and prolonged illness requiring surgery in 3.

The USP vaccine, given in doses exceeding 3×10^9 killed plague bacilli, capable of stimulating MPI below 5 in as many as 75 percent of human subjects, caused unpleasant local reactions of varying incapacitating intensity and occasionally severe systemic reactions, with fever, myalgia, headache and even vomiting.

(B). Killed Plague Vaccines with Synergists.

From 1939 to 1948, extensive experiments on small laboratory animals had demonstrated that chemically killed (with formaldehyde or alcohol) and partially detoxified, virulent plague bacilli, grown at 37°C and precipitated with alum, invariably conferred upon guinea pigs a slightly superior protection against infection than did the standard vaccine. Since these preparations induced persistent local reactions in animals and unpleasant side effects in human subjects, further studies with this type of vaccine were discontinued. In

1960, Cutter Laboratories prepared several batches of vaccine by coating the individual plague bacilli with aluminum hydroxide. Two production lots, consisting of 1.6×10^{-9} ml formalin-killed P. pestis 195/P (0.471 mg N/ml) and 3.2×10^{9} bacilli (0.73 mg N/ml), were evaluated on mice, guinea pigs, rabbits and nonhuman primates. In the mouse protection test, the alum vaccine proved 4 times better than the standard one as a protective antigen in guinea pigs. Basic immunization with 4 or 2×10^{-9} vaccine, followed by a booster of 2×10^{9}, conferred resistance to potentially fatal subcutaneous challenge (but not to local infection). Protection against fatal respiratory or intraperitoneal infection was equally demonstrated in 70-80 percent of the guinea pigs so inoculated; death was frequently delayed as long as 2 weeks. A single vaccine inoculation, followed by a booster in the 3rd or 4th month, completely protected Macaca mulatta against an intraperitoneal infection which proved fatal to the nonimmunized monkeys. Clinical tests on human subjects confirmed the fact that the large (3.2×10^{9}) dose of alum-adsorbed plague vaccine inoculated intramuscularly caused local reactions in 83 percent and general reactions with fever in 33 percent. The antibody response was of a low order, thus confirming the observation that a single basic inoculation with an adjuvant vaccine does not produce significant immunity. A booster inoculation of 0.2 ml (8.0×10^{8}) raised the number of MPI below 10 to 60-66 percent. In another test, with the basic inoculation of vaccine containing 1.6×10^{9} P. pestis, local reactions occurred in only 7 percent of the men but rose to 16 percent with booster inoculations of 0.1 ml. Judging from the levels of the mouse protection indices after basic and booster inoculations, the synergist vaccine in small doses of antigenic mass was as effective as the large dose preparation. The importance of the synergist was recognized in one trial, when the antibody responses of control groups of human subjects receiving single inoculations of 1 ml of $Al(OH)_3$ and those of others given an old type phenolized saline vaccine were compared. Prebooster (6 months) and postbooster MPI levels below 10 appeared quickly and extended from 23 percent to 66 percent of the human subjects inoculated with alum-adsorbed antigen. In comparison, in the group given saline antigen (in 1 ml doses), significant levels appeared in 6 percent before booster and in 29 percent after booster. Second and third booster inoculations, with or without synergist, adjusted the differences; in both groups, approximately 64 percent of the human subjects acquired significant protective antibodies (Reports 13, 14 and 15, and histogram chart in Report 18, p. 31). A clinical trial using a synergist alum-adsorbed USP vaccine proved disappointing. Shortly after preparation of the vaccine, the mouse potency test yielded a ratio of 10, while that of the aqueous USP vaccine was 4.0. Both preparations had been held for 9 months at ±4°C before use in the clinical trial. On the 28th day after intramuscular inoculation of 1 ml of the alum-adsorbed vaccine, only 20 percent of 24 human subjects had MPI below 10 and there were none below 5. This vaccine likewise proved inferior in booster efficacy. Only 17 percent of the human subjects had MPI below 5; 60 percent were at a level of 10 or above (Report 18). While the alum-adsorbed killed plague vaccine has a relatively short shelf-life, an aluminum-phosphate-precipitated preparation proved stable for at least 3 years, and, in the dosages of 350 to 900 million organisms, protected 70-80 percent of groups of guinea pigs treated with single doses and challenged 3 weeks or again 3 months after inoculation with 700,000 virulent P. pestis (Report 13). Further clinical trials were deferred until the problems of manufacturing stable, synergist-killed plague vaccines have been solved. The USP vaccine is the only plague prophylactic available for mass inoculations of Armed Forces personnel.

(C). Combined Plague-Cholera-Typhus Vaccine Designated "Exotic Package".

It was suggested by the Commission on Immunization that a combined plague-cholera-typhus suspension, the so-called "exotic package vaccine," be prepared and tested on animals and human subjects to determine the character of local and systemic reactions and whether a single 1 ml inoculation of the combined antigens has a significant primary action for subsequent booster response. It was decided that the experimental vaccine should contain:

2×10^9	formalin-killed virulent plague bacilli coated with 0.08 percent Al (OH_3)
8×10^9	Vibrio cholerae; and
0.1 ml	whole commercial high-potency Cox-type typhus vaccine.

Since plague vaccine in the proper concentration of 0.5 ml was available and commercial typhus vaccine was readily obtainable from Lederle Laboratories (courtesy of Dr. H. Cox), it was only necessary to procure the specially prepared phenol-killed Vibrio cholerae which contained 8×10^9 in 0.4 ml suspension. Dr. J. O. MacFarlane (Technical Associate, Biological Development, Eli Lilly and Company) kindly provided and prepared the desired cholera vaccine, Lot I-58526, containing 20 billion cholera organisms equally divided between the Inaba and Ogawa strains, per ml suspension comprising 0.08 percent aluminum hydroxide and 0.5 percent phenol.

The final "exotic" mixture, compounded by Cutter Laboratories and subjected to safety tests, was remarkably well tolerated in several species of laboratory animal and produced no local nor systemic reactions. Potency tests for plague antigens fulfilled the accepted standards for regular Army vaccine. Potency tests for typhus, conducted by Lederle Laboratories, and for Vibrio cholerae antigens by Eli Lilly and Company, indicated that the vaccines were potent. The combined antigen vaccine proved highly protective against plague for Indian Hanuman langurs but not for Malayan silver leaf langurs. In man, it produced the same percentage of local reactions as typhoid vaccines, but it caused fever in 1 subject (2 percent) and general malaise in 12 or 26.6 percent of the men -- more than a twofold increase in the frequency anticipated from previous tests with the usual monovalent plague vaccine. Twenty-eight days after inoculation, all sera revealed hemagglutinin titers varying from 1:4 to 1:128, slightly better than those recorded in previous trials on man with alum-coated, formalin-killed virulent plague vaccine. Mouse protection indices (MPI) were below 10 in 27.7 percent of the subjects; only 5/36 or 8.3 percent had MPI below 5. A 2 ml booster dose 3 months after basic inoculation was only moderately effective. Although several individuals acquired fairly high passive hemagglutinin titers, 5 men failed to react. Furthermore, only 17/36 (45 percent) yielded MPI below 10, with 5/36 or 13.9 percent disclosing MPI of less than 5. The test for typhus antibodies, conducted by Dr. Charles L. Wieseman, University of Maryland, suggested that the typhoid vaccine component was not of highest potency. Only 10/36 or 27.7 percent of the vaccinated subjects yielded toxin-neutralizing antibodies after booster inoculation. Agglutination tests for cholera were performed at the Walter Reed Institute for Medical Research under the supervision of Dr. E. Becker, using Vibrio cholerae Inaba and Ogawa. Approximately 3/4 of the sera showed 4- to 10-fold rises in specific antibody titers 1 month after basic inoculation. Elevation of antibodies was induced by the small booster dose inoculation (only 1,600 million Vibrios) 3 months after basic immunization. The 1:16 titers were doubled, while titers of 1:32 were nearly tripled. The significance of these rises, possibly reflecting an improved state of immunity, cannot be appraised by means of the existing knowledge of the antibody complex.

In summary, an experimental combined plague-cholera-typhus vaccine, empirically compounded from available commercial antigens, produced no undesirable side effects but proved slightly less immunogenic for plague than the standard monovalent vaccines. The shelf-life of the vaccine is unknown. Studies to standardize the quality and potency of the combined vaccine are essential before further trials can be instituted (Report 17).

(D). Haffkine Vaccine.

During the WHO discussions considering field trials to evaluate the protective efficacy of killed plague vaccine, comparison of the USP and the Haffkine appeared to be indicated. The latter vaccine had never been tested using the procedures developed by the G. W. Hooper Foundation; in particular, the antibody response following single and booster inoculations had never been assessed. The past history of the vaccine emphasized the severity of local and even systemic reactions. Personal consultation with WHO representatives and the Director of the Haffkine Institute in Bombay (Dr. N. K. Dutta) resulted in the preparation of a program for investigations. The Institute will test the protective properties of its vaccines by infection experiments on langurs similar to those evaluating USP vaccines grown on E medium (Report 18, pp. 13-17). While these tests are in progress, clinical trials on inmates of the California Medical Facility, Department of Correction, Vacaville, California, are being conducted.

Samples of the 2 types of Haffkine vaccine were furnished from Bombay. In recent years, there have been some changes in the procedures involving manufacture of the vaccine. The current preparation, now produced from the fully virulent strain P. pestis 195/P, is grown by means of a rotary-shake culture method at 36° or 37°C in 37 H (plain casein hydrolysate base) medium in the presence of adequate calcium content for 48 hours. The cultures are killed with a final concentration of 0.07 percent formalin at the end of the growth period. After collection of a sample for purity tests, the vaccines tested for sterility are preserved with 0.0015 percent phenyl mercury nitrate. The final vaccine is assayed by the standardized mouse protection test of Sokhey and Maurice (1939). Extensive comparative tests proved the new vaccine to be as potent as the old Haffkine, which was prepared in plain casein hydrolysate with 14 days' incubation at 32°C. There is no apparent difference in the toxicity of the old Haffkine Institute vaccine and the vaccine prepared at 37°C in shake cultures; mice tolerated doses of up to 0.4 ml, but with 0.6 ml of either vaccine, a number of mice died (Gadgil et al. 1967). The results of a comparative mouse protection test performed by the G. W. Hooper Foundation on Swiss Webster mice from BioScience Laboratory indicate that the old and new Haffkine vaccines have approximately the same protective efficacy as the USP (Cutter) vaccine and the NIH Biological Control Reference Number 2 standard vaccine.

Batch of Vaccine	PD_{50}	Ratio	PD_{50} value in ml
Haffkine Lot 27-7 (old)	1:235	1.2	0.00168
Haffkine Lot 27-2 (new)	1:250	1.2	0.00160
USP Cutter K5239	1:175	0.9	0.00228
NIH Ref. No. 2	1:195	-	0.00204

In the protection or potency test on guinea pigs, the Haffkine vaccines proved slightly inferior to the USP (Cutter) vaccine; groups of 20 guinea pigs, each inoculated subcutaneously with 1 ml and subsequently (on the 20th day) with 0.2 ml of the different preparations, were challenged with 1,280,000 virulent P. pestis 195/P on the 30th day after the last injection. The USP vaccine protected 20/20 (100 percent) of the vaccinated guinea pigs; the old Haffkine; 14/20 (70 percent), and the new, 12/20 (60 percent). When emulsified in arlacel and Drakeol, the Haffkine vaccines improved slightly in potency and protected 17/20 (85 percent).

Twenty-five California Medical Facility (Vacaville) inmates without histories of exposure to plague or plague antigens, and 9 who had been included in clinical trials with live plague vaccines (E.V. 51f), were each inoculated in the deltoid muscle with 1 ml of either the old or new Haffkine vaccine. On the 24th hour after inoculation with either type of vaccine, local reactions were definite in the vaccinated group. Erythema varying from 5 to 9 cm patches and increased warmth and tenderness of the indurated areas were noted in 4 inmates (see Banker 1968). These expressions of hypersensitivity were accompanied by pain radiating to the axilla. Two men complained of slight malaise in the form of headaches. There were no elevations in body temperature, but in 5 individuals (5/9) the WBC rose (average 7,500-11,400), persisting for 48 hours in 3 subjects. No lymphadenopathies were palpable. In 2 previously inoculated inmates, the warm, indurated intramuscular areas persisted on the 6th day after vaccination, accompanied by malaise and discomfort from erythema and edema spreading for 10 cm over the deltoid region and upper part of the biceps muscle. In the previously nonvaccinated groups, the absence of erythema, regardless of the type of vaccine administered, was noteworthy in all but 2, who exhibited 2 x 2 cm areas of faint redness. Increased warmth of the skin or tenderness on touch indicated the location of small indurations causing no impairment of function. Two inmates, who had resumed hard physical labor immediately after inoculation, had apparently thus dispersed the antigen through the deltoid muscle, which became tender, with a painful, hot, indurated area at the lower tip, accompanied for 24 hours by axillary pain which subsided by the 48th hour. Some subjects complained of malaise, slight headache, and nausea, but there were no elevations in body temperature. Fourteen (14/25) had definitely increased WBC (from an average of 7,300-12,000;

highest, 18,000); the leukocytosis disappeared by the 48th hour in 10 men, but reappeared in 2 on the 2nd day after vaccination. Three complained of malaise with fleeting headache. The reactions, local and systemic, in contrast to those following live vaccine inoculation, were exceptionally mild. In fact, they were less marked than those elicited by the USP vaccine. Certainly the vaccine, which has been produced in India since 1945 in casein-hydrolysate media and formalin-killed, when used intramuscularly in the dosage of 1 ml, does not cause the notorious unpleasant local and systemic reactions which accompanied subcutaneous 2-4 ml inoculations of the original "vaccine brew" prepared according to the method of Haffkine (Report 18, p. 32).

Serum antibody measurements conducted on the 28th day after basic inoculation revealed that the new Haffkine vaccine, perhaps by mere coincidence, produced a higher percentage of mouse protective antibodies below 10 than did the USP (40 percent versus 18 percent). On the other hand, average passive hemagglutinin titers induced by the Haffkine are lower than those elicited by the USP vaccine. A single inoculation of a killed or even a live plague vaccine confers little or no indirect evidence of protection. Repeated re-inoculations are essential to produce significant mouse protection indices.

The clinical trial included 9 inmates who had previously received small doses of live attenuated P. pestis E.V. 76 (51f passage) and E.V. Paris. All experienced unpleasant side effects, but the antibody response, expressed by MPI below 10, was evident in 3/4 inmates inoculated with 1 ml doses of the new Haffkine, whereas 0/4 vaccinated with the old Haffkine exhibited such antibody response. Thus it is noteworthy that live plague vaccines, even in relatively small doses, sensitize the immunity mechanism in man.

The search for a specific protective antigen, so vigorously pursued in 1940, lost incentive when it was realized that complex cell wall preparations in dosages causing no local reactions in guinea pigs rarely protected more than 65 percent of this species (Report 17, pp. 13-15, Table 5). Furthermore, Fraction I administered in booster schedules stimulated the appearance of HA, low mouse protection antibodies, and evidence of pronounced hypersensitivity, but conferred no protection upon 4/6 (Death/Total) langurs or 4/6 African vervet monkeys against subcutaneous virulent infection. Although death was delayed, it was invariably complicated by extensive necrotizing dermatitis and regional lymphadenitis and toxemia, with hemorrhages and invasion of the blood-stream by intestinal flora. It was deemed inadvisable to analyze the protective efficacy of various plague antigens until the immunogenicity of whole P. pestis had been evaluated in several animal species and particularly in man by means of clinical studies. Correlations derived from clinical trials on man are essential to establish the validity of the laboratory-assay methods which have been developed. As outlined above, some progress has been achieved, and the limitations of USP killed plague vaccine may now be listed: (1) A single inoculation of the prophylactic (1 ml = 2 billion organisms), whether consisting of correctly prepared killed vaccine with or without a synergist, or chemical antigens in dosages causing little or no discomfort, never stimulates demonstrable serologic immunity in the form of hemagglutinins and mouse protective antibodies (below 10) in more than 30 percent of individuals inoculated. (2) After booster inoculation with a small dose (0.2 ml), significant antibody titers appear in 60-66 percent of the inoculated men. (3) In fact, limited observations indicate that these hemagglutinins appeared and persisted in 100 percent of inoculated human subjects provided they had received 6 or more booster inoculations. (4) Re-inoculations with killed vaccines recall serum antibodies in men previously vaccinated 10 to 20 years ago.

Unfortunately, our present knowledge of the efficacy of killed plague vaccines ends at this point. Carefully controlled field trials have not been conducted. During the past 5 years, the killed USP vaccine has been used in mass inoculations, necessitated by the fact that US Army personnel have been operating in plague areas which became highly endemic under war-time conditions.

Published reports (D. C. Cavanaugh et al. 1968) emphasize the alarming frequency of plague in the Republic of Vietnam since 1962. Indeed, it is stated that 13,263 or 45 percent of a total 29,109 known cases in that territory since 1906 have been reported as occurring in the last 5 years. Under existing conditions in Vietnam, the only measure available to most of the population for personal protection against plague is vaccination. Data on the efficacy of the living plague vaccine (E.V. strain) employed by the Government of Vietnam are lacking, but a marked reduction of human plague has been noted in areas where intensive vaccination campaigns have been executed.

US Army personnel inoculated with USP vaccine enjoy almost complete freedom from plague, despite their frequent exposure. One of the 4 known and confirmed cases of bubonic plague among North Americans occurred in an unvaccinated individual. Two confirmed cases of pneumonic plague and 1 case of asymptomatic pharyngeal plague have been recorded among individuals inoculated with the USP vaccine. However, it should be noted that US Army personnel are protected, whenever possible, by much higher standards of environmental sanitation than those available to the average Vietnamese in the same area.

A group of expert clinicians familiar with the techniques of treating lymphocytic blastogenesis and hypersensitivity are using plague antigen inoculations as a model for special immunological studies. This group has observed that whenever a volunteer experienced severe local and systemic reactions to the vaccine, he exhibited the highest *in vitro* lymphocytic response to Fraction I. In 9 of 12 human subjects, gross, apparently positive, delayed hypersensitivity to Fraction I developed on the 7th day after immunization. Some individuals produced 19S and 7S globulin as early as the 7th day.

In conclusion, the following conservative statement appears to be justified: Recent experiences in Vietnam indicate that persons vaccinated with killed plague vaccine enjoy almost complete freedom from the disease, although frequently exposed to infection (Meyer 1970).

VIII. The Present Status of Live Attenuated *P. pestis* Vaccines.

Due to the impact of discussions at the WHO Traveling Seminar in the USSR (Section VI of this report, p. 50), it appeared imperative to restudy the efficacy of living plague vaccines. Since the primary objection to the use of live vaccines centers on the viability, discomfort and possible harmfulness engendered by side effects, research first focused on the preparation of stable, freeze-dried, live preparations with a high percentage of viable cells possessing all the antigens thus far identified. Second, cutaneous or intracutaneous administration procedures were studied to determine the minimum number of viable cells required to induce in man a benign immunity process resembling a very mild infection with no unpleasant local or systemic reactions and resulting in a relatively stable resistance comparable to that of individuals who have contracted and recovered from plague. Third, antibody response must be measured, with special reference to hemagglutinin and mouse protective antibodies, in highly susceptible nonhuman primates and then in man.

(A). E.V. Saigon (WR-S) Vaccine.

By courtesy of the Walter Reed Army Institute of Research, Washington, D.C., vials of a freeze-dried vaccine, prepared with the E.V. isolate of the Institut Pasteur, Saigon, became available. The vaccine (E.V.S) was grown on agar at 28°C and suspended in a stabilizer consisting of 0.2 M phosphate containing 7.5 percent sucrose, 0.7 percent glutamate and 2.5 percent human serum albumin. The contents of the vials, comprising dead bacilli and approximately 23-40 percent viable *P. pestis*, were reconstituted with sterile water.

The protective dose for mice varied from 2500 to 2800 viable organisms. A dose of 0.1 ml vaccine (consisting of 40,000, 4000, 400 or 40 viable P. pestis E.V.S) inoculated intradermally into guinea pigs produced erythematous areas (1.5 cm in diameter) with yellowish pinhead-sized nodules, which converted to eschars and then to scars by the 12th day. Lymphadenopathy was slight but evident. The immune response, measured by hemagglutination, complement fixation, and mouse protective antibodies, was significant: out of 18 guinea pigs each vaccinated with 400 to 40,000 organisms, 8 had MPI below 10 on the 28th day. Challenged with 1,250,000 virulent P. pestis subcutaneously or 8000 intraperitoneally doses containing 400 to 40,000 viable E.V.S organisms protected 100 percent of the guinea pigs against subcutaneous infection and 97 percent against intraperitoneal infection. Doses with 40 viable P. pestis protected 80 percent of the animals against subcutaneous infection but only 10 percent against intraperitoneal infection. A freeze-dried living E.V. from Stavropol in the large doses conferred identical protection; the lowest dose (34 bacilli) immunized 30 percent and 20 percent against subcutaneous and intraperitoneal infection, respectively.

Groups of Hanuman langurs (Presbytis entellus), vaccinated intradermally with 17,800, 178,000 or 1,780,000 viable E.V.S organisms, exhibited erythematous areas (5 mm x 10 mm) with indurated centers (2 mm x 2 mm) which persisted for 3 to 4 days. The regional lymph nodes were palpable but not strikingly enlarged. Antibody response was vigorous; all had MPI below 10 (5/10 (50 percent) below 5) before challenge. Re-infected with an LD_{50} of 1000 (232,000) virulent P. pestis, only 5/11 (45 percent) survived, while 6/11 (55 percent) died within 7-19 days (controls died within 3-10 days), with gross pathologic and bacteriologic manifestations of bubonic-septicemic plague. Only 2 of 5 macaques intradermally vaccinated with single doses (514 million) of E.V.S organisms survived intraperitoneal challenge, but in another group of 5 macaques similarly vaccinated on the same day and each boostered with 82,000 viable vaccine organisms, all survived intraperitoneal challenge with 7 million virulent P. pestis 195/P (fatal to 4/5 controls on the 3rd to 5th day).

Groups of 8 langurs, each vaccinated with either 42 million or 420 million viable E.V.S organisms, responded exceptionally well with antibodies; in all but 2, the MPI was below 5 on the 28th day. By the 406th day after basic vaccination, booster inoculations given to one-half of each group had restored the serologic reactions. Finally, on the 85th day after booster inoculation (491st day after basic inoculation), the 15 langurs still alive were challenged with virulent P. pestis (fatal to all controls); 10 animals survived. Of these 10 immune langurs, 6 belonged to a group of 8 given 1 booster inoculation each, while 4 belonged to a group of 7 not given boosters. Despite the heterogeneous reactions of langurs, the experiments proved that the immunity induced by 42 million live E.V.S organisms persisted for at least 16 months and that a small booster inoculation maintained the resistance.

Two clinical trials with E.V.S organisms on human subjects were disappointing: (a) In the first pilot experiment, intended to measure the pathogenic effects of this vaccine, groups of 5 men were inoculated subcutaneously with 535,000, 5,350,000 or 53,500,000 viable organisms; only the medium and large dose injections produced erythema persisting for 48-72 hours. In the highest concentration of 53 million bacilli, the vaccine failed to sensitize or to stimulate MPI. No booster dose effects were elicited with either the live vaccine (4.5 million viable organisms) or 1 ml killed alum-adsorbed Cutter plague vaccine. (b) Subcutaneous vaccination with 420 million viable freeze-dried E.V.S organisms induced pronounced erythema and some induration for 96 hours. Two men had severe local reactions, leading to abscess formation in one of them; 6 (24 percent) had fever, and 7 (28 percent) revealed leukocytosis. All subjects had slight lymphadenopathy. Some individuals found the side effects distinctly unpleasant but milder than those produced by the 1951 vaccine E.V. 76. Hemagglutinins in low titers but no complement fixing or mouse protective antibodies were evident, even after booster inoculations with 8.45 million organisms of the same vaccine; the vaccinated men all failed to produce mouse-protective antibodies.

Similar trials on human subjects by other investigators demonstrated that mass vaccination with a subculture of an E.V.S strain possessing all the biochemical, nutritional and marker determinants apparently invasive and immunogenic for guinea pigs, mice, langurs and macaques and capable of inducing significant antibody levels, produced unpleasant local and systemic reactions upon intracutaneous injection.

In response to booster inoculations with Fraction I or killed, alum-precipitated vaccine, only 5/12 human subjects (41 percent) demonstrated mouse protective antibodies considered adequate to confer relative active immunity against plague infection, even after primary inoculation of 420 million organisms. Further studies with the E.V.S strain have been discontinued until the complex factors responsible for such disparities are comprehended.

(B). E.V. 76 Paris:

The disconcerting experiences with the P. pestis E.V. Saigon vaccine logically necessitated testing a transplant of the original culture. By courtesy of Dr. Girard, a subculture of his E.V. 76 strain of P. pestis maintained at the Institut Pasteur, Paris, was obtained in December 1967. The PD_{50} for guinea pigs vaccinated intracutaneously was 40 viable organisms by intraperitoneal challenge and 33 viable organisms by challenge via the subcutaneous route. Even in a dosage of 12.1 billion organisms, the E.V. 76 (Paris) strain proved nonpathogenic for guinea pigs when inoculated by the subcutaneous route, and 100,000 bacilli were nonpathogenic for mice by intravenous inoculation; but the PD_{50} was 38 viable organisms by intraperitoneal challenge and 2800 viable organisms by challenge via the subcutaneous route.

The transplant of the original E.V. 76 stored in the Institut Pasteur for nearly 35 years has retained all the biochemical determinant characteristics and immunogenic properties for guinea pigs defined by Dr. Girard and others. A vaccination experiment on 4 langurs, 6 vervets and 4 macaques each inoculated intradermally (left femur) with 26,800,000 viable E.V. 76 organisms is informative. Regardless of age and weight (8-15 kg), local reactions were observed in the langurs in the form of erythematous indurated areas (5 cm x 5 cm) and prominently enlarged, firm but movable, regional lymph nodes (up to 4.5 cm x 2.5 cm), which receded very slowly by the 25th day. These local responses were accompanied by systemic reactions; fever, anorexia, and loss of aggressiveness were noted in all the animals, and positive blood cultures persisted until the 8th day in 1 young langur. Lymph node biopsy specimens revealed confluent growth of E.V. 76 (Paris) organisms. Recovery was protracted.

Systemic reactions were less marked in the vervets, although slight elevations in temperature (up to 40ºC) for 1 or 2 days were recorded and positive blood cultures were obtained on the 5th day from 2 animals. Cutaneous inflammatory nodules (1 cm x 1 cm) subsided within 12 days, but the inoculation sites remained visible as eschars or desquamated, hairless areas. Inguinal lymphadenopathy, however, in the form of hard but movable nodes varying from 2 cm to 3 cm in size in some individuals, accompanied by moderate enlargement of the opposite inguinal nodes, resembled the reactions observed in langurs. On the 5th day, biopsy specimens from the enlarged lymph nodes of 4 animals yielded abundant growths on blood plates. Smears prepared from biopsy aspirates revealed unevenly distributed clusters of typical P. pestis. As in the langurs, the enlarged nodes persisted for more than 30 days.

Local reactions in the macaques were confined to pinhead-sized pink nodules which were reduced to hairless areas (5 cm x 5 cm) by the 8th day. Regional lymphadenopathy in the form of firm but movable nodes varying in size from 1 to 2.5 cm was similar to that observed in this species vaccinated with strain M_{23}. Biopsy specimens of the largest nodes on the 5th day yielded no cultures. The animals in this group were active, but after exercise occasionally exhibited 1-2^{0} rise in temperature. One animal had diarrhea on the 3rd day and a positive blood culture on the 5th day; it died on the 8th day. The autopsy findings were atypical for plague, apparently complicated by a small pneumonic area of Klebsiella, and nonspecific gastroenteritis; nevertheless, E.V. (Paris) organisms were isolated from the heart blood, left and right inguinal and enlarged left iliac lymph nodes, spleen, liver and bile.

The serologic study was conclusive in respect to antibodies: langurs, vervets and macaques had MPI of 3 to oo, hemagglutination titers of 1:512 to 1:4096 and complement fixation titers of 1:4 to 1:32. This experiment disclosed the E.V. 76 strain extensively used in human mass vaccinations to be highly invasive and pathogenic for 3 species of nonhuman primates. Upon challenging langurs with doses of 343,000 virulent P. pestis 195/P organisms, vervets with 155,000 subcutaneously, and macaques with 7.5 million intraperitoneally, the 4 langurs, 4/6 vervets, and 2 macaques proved solidly immune, with no local reactions at the sites of infection. Two vervets, however, revealed local lymphadenopathies with draining skin pustules discharging P. pestis for up to 9 days. Nonvaccinated controls all died on the 4th day. Finally, it bears repeating that the E.V. (Paris) isolate acquired increased "virulence" with passage through guinea pigs. The passage isolate (E.V. 76 Paris f) has an LD_{50} of 3000 or less upon intravenous inoculation in mice, in contrast to the original subculture, 100,000 viable organisms of which caused no deaths. Thus E.V. Paris is potentially harmful.

A clinical pilot trial explored the theory that the freeze-dried E.V. Paris isolate in small doses administered by the cutaneous-puncture method might prove immunogenic without causing unpleasant side effects. A group of 9 inmates of the California Medical Facility of the Department of Correction, Vacaville, were inoculated by the bifurcated needle technique. One group of 5 inmates had previously (1967-68) been inoculated with killed USP plague vaccine, and all had responded with HA and mouse protective antibodies. The second group of 5 men had no histories of previous vaccination. Since Lot 2 of the freeze-dried E.V. 76 Paris vaccine had a relatively low viability (probably due to deterioration from lyophilization and ineffective storage procedures), the average number of viable cells in the reconstituted vaccine adhering to the bifurcated needle varied between 300,000 and 490,000 (average 406,000). Two inmates were given single punctures, while 3 were each given 4 punctures (1,600,000 *P. pestis* organisms). Daily observation, as well as photographs of the local lesions, disclosed erythematous nodules 2-5 mm in diameter, gradually increasing to 7 and 9 mm by the 96th hour after vaccination. Small eschars developed at the puncture nodules and dropped off by the 10th-12th day. By the 76th hour after vaccination, no febrile reactions had been recorded, but there was a slight elevation in the WBC of 1 inmate. No axillary lymphadenopathy developed in any of the vaccinated men. The verdict of those who observed the reactions was unanimous that, despite the temporary discomfort of the puncturing, reactions were less marked than those induced by the intracutaneous syringe method with E.V. WR (Saigon strain) (Report 18). Serologic tests *in vitro* and *in vivo* disclosed that with one exception -- a previously vaccinated inmate -- none of the men demonstrated hemagglutinins. The mouse protection indices (average 15 to 16) remained essentially the same -- no booster effect was noted after inoculation. In view of the finding that 130,000 E.V. 76 Paris organisms are required to stimulate highly significant HA titers and reduction of the MPI in guinea pigs, a dose of 1,600,000 organisms would seem inadequate to stimulate even a very sensitive immunity mechanism to produce hemagglutinins. This deduction was tested in 2 separate clinical trials.

(1) Ten inmates who participated in the preceding trial and 4 men not previously exposed to plague were inoculated cutaneously with E.V. 76 Paris G (Lot I) in an average dose of 28 million viable organisms administered in 4 punctures by bifurcated needle. Cutaneous reactions consisted of either merely needle puncture-marks with small brownish center crusts or erythematous nodules 10 mm in diameter, appearing by the 24th hour. By 48-72 hours, the slightly raised, tender nodules or papules (10-24 mm in diameter) were surrounded by individual inflammatory rings or embedded in confluent 40 x 40 mm, faintly erythematous areas. On the 72nd hour, and more definitely on the 96th hour, the local lesions had acquired a vesicle-like character, consisting of erythematous zones surrounded by narrow, yellowish, slightly raised rings with central brownish crusts. Generally the peripheral erythema had faded by the 6th day and the eschars, removed by shower-baths, had left slightly glistening center rings which had converted to pale scars by the 14th day. Axillary lymph nodes were not palpable. Despite complaints of flushing or "flu reactions," no elevations in body temperature or changes in WBC were recorded. In the previously vaccinated group of inmates, the evolution of local reactions to 28 million E.V. 76 Paris organisms was essentially identical to that in the control group, with 2 exceptions: by the 48th hour, papule-vesicle formation was already

marked in 2 men. In one, the temperature had risen to 99.9^{0}F by the 28th hour and the WBC was over 10,000. The serologic results in terms of antibody production -- hemagglutinins (determined by 3 different laboratories) and mouse protection indices (MPI) -- in a group of 4 inmates vaccinated with killed and small doses of live vaccine and in 5 inmates given only live vaccine, reflected low titers in response to cutaneous multiple-puncture vaccination. On the 30th day after contact with 28 million organisms, 1 previously vaccinated subject (MPI 2) and 1 man with no history of contact with the plague bacillus reacted with the significant MPI titer of 7. This disappointing immunogenic response has interesting implications and thus deserves brief comment.

The punctures, intended to accurately introduce approximately 28 million E.V. Paris organisms into the skin of each inmate, were performed with particular care. Experiments on guinea pigs and nonhuman primates with dilutions of the inoculate had confirmed the accuracy of the technique employed. A study of local reactions at the puncture sites disclosed variations. Within 6 to 8 hours, the sites of the needle-punctures were surrounded by raised marked erythematous rings; the diameter of the vaccine reaction area (measured by calipers) was 10-12 mm in the men not previously vaccinated, while it was generally smaller in the vaccinated (except in 2 individuals, where the inflammatory reaction to the vaccine was combined with or overshadowed by delayed hypersensitivity). The inflammatory nodules, quite pronounced by the 48th hour, changed to papules or vesicles which converted to brownish crusts by the 8th-10th day; the dropping of the eschars had left pale scars by the 14th day. The subjects described these local reactions as "ugly," although they did not interfere with arm function. The absence of palpable axillary lymph nodes was particularly interesting. Apparently the local inflammatory macrophage reaction immobilized the inoculated P. pestis in the skin and prevented transport of the invasive mutants to the lymph nodes. Thus in all probability, few antibody-producing cells were brought into contact with unaltered antigen. In fact, it is suspected that the antigenic mass reaching the circulation was inadequate and therefore failed to exert a definite booster effect on the mouse protective antibodies, but raised the hemagglutinins in some individuals. Clinical observations on the local reactions which follow the intracutaneous inoculation of E.V. 76 vaccine suggest that the inflammatory skin-tissue response represents some form of defense against infection. Since (according to Girard and others) a "mild infection" with live vaccines is essential to produce protective immunity, it is a matter of conjecture whether the cutaneous or intracutaneous methods of administration, recommended to avoid unpleasant side effects, are effective with relatively small doses. The "local immunity" (if this designation may be used in the absence of general immunity) may be partially due to man's resistance to plague, which is slightly higher than that of langurs, guinea pigs and mice. The toxins in the dead plague bacilli - 60-70 percent in the average lyophilized live vaccine - obviously enhance the inflammatory reaction and promote local mobilization of body defenses.

(2) To test official reports that large doses (at least 400-500 million organisms) of viable E.V. 76 are required to confer protection, another clinical trial was conducted. In order to avoid the more severe local cutaneous reactions, the vaccine was administered by the subcutaneous route. A group of 12 men, composed of 5 previously vaccinated with USP vaccine and 7 with no previous exposure to plague, were each inoculated subcutaneously with 56 million viable P. pestis E.V. 76 Paris (freeze-dried Lot I, G-WRAIR). As anticipated from previous experience, local and, to a lesser extent, systemic reactions were quite marked. By the 24th hour, faintly red areas, which by the 48th hour had become bright red, slightly indurated inflammatory zones varying in diameter from 60 to 120 mm, some spreading over the upper arm, had caused discomfort and tenderness to touch but no impairment of function and few or no complaints of malaise. Beginning on the 72nd hour, fading of the erythema disclosed nodular indurations at the inoculation sites. By the 144th hour, the erythema had disappeared but in 3 individuals (who described it as "itchy"), the area was covered with fragments of desquamated skin. Palpable nodules persisted for 2 to 3 weeks. The general consensus concerning the vaccinations was a unanimous declaration that the side effects were unpleasant and more pronounced than those previously experienced with the USP vaccines tested at the California Medical Facility. Painless, slightly enlarged lymph nodes were palpable in the axillae of 10 human subjects. The body temperature of 1 individual had risen to 99°F on the 1st 2 days after vaccination, while the WBC were above 10,000 (11,000-14,000) within the 1st 24 hours in 10 men. The WBC remained the same in 8 of the men by the 48th hour, but in only 2 by the 72nd hour, and then once again ascended on the 19th day. In previously vaccinated inmates, passive hemagglutinin titers of 1:128, present on the 6th day, had risen significantly by the 14th-20th day; but these titers tended to decline by the 30th day. Simultaneously, the MPI had dropped to levels below 5 in 3/4 men by the 14th day, but had risen again by the 30th day. In contrast, among the individuals not previously inoculated, the HA titers of 5/7 were initially 1:16 to 1:32 and eventually reached about 1:64 to 1:512. The average normal MPI of the nonvaccinated subjects, established as 16 (14-18), had

dropped to 13 by the 30th day, and in 1 man to below 10, with a corresponding HA titer of 512. The following conclusion appears to be justified: The live E.V. Paris vaccine exerted a significant booster-dose effect in previously vaccinated human subjects, while the antibody response in the nonvaccinated -- with 1 exception -- was similar to that induced by a single dose of USP killed vaccine (average MPI 13) on the 28th day after inoculation (see Report 18, Table 2, p. 14). In order to attain MPI indirectly expressing protection against plague, a 2nd booster inoculation is apparently required. Nearly identical observations had already been reported in 1953 (Meyer, Bull. WHO and Report 3, Table IX, p. 36); the average MPI was 12 in human subjects following single inoculations of 1 billion (350 million viable) organisms of the E.V. 76 isolate causing severe local and systemic reactions, then in use in Madagascar. Booster inoculation with 1 mg Fraction I brought the average MPI to 8.6, and in 3 subjects to 1 or 2. On the basis of a more extensive series of studies, Payne and his associates (1956) concluded that the initial inoculation of living attenuated *P. pestis* strain E.V. elicits demonstrable Fraction I antibodies (HA) and antitoxin in relatively few persons. Repeated vaccination, however, enhances the production and maintenance of these antibodies by individuals and groups. A search of the available Soviet literature produced documentation that the HA test (Konikova *et al*. 1969) but not the mouse protection test has been used to measure the response to live plague vaccine in man. The observations of Aleksandrov and his associates (1961) on small groups of human subjects indicated that average hemagglutination titers on the 30th day following single subcutaneous doses were of a relatively low order (1:92), but were raised to 1:203 by a 2nd inoculation. As might be expected in view of the results detailed in the report, the low titers (1:60) which appeared after cutaneous vaccination (1:52) were not improved upon re-inoculation by the same route. Both the Soviet and French literature dealing with innumerable repetitious experiments on guinea pigs and occasionally on mice is singularly devoid of attempts to measure immunity except by challenge infection. In 1956, Korobkova, in her classical treatise "Live Antiplague Vaccine," said (in translation): "Up to the present time no reliable test exists for characterizing the specific changes of reactivity of the body which set in after the inoculation of live plague vaccines. Single and even double vaccination are not accompanied by notable accumulations of antibodies on the basis of which it might be possible to evaluate the immunological composition of the vaccine; no agglutinins, precipitins, bacteriolysines or other immune bodies are found which might attest to the onset of immunity, despite the fact that the animals immunized with the live vaccine acquire a great degree of resistance to the plague infection and tolerate the infection by large doses of the virulent microbes." In his latest review, Girard (1963, p. 44), reviewing the work of Spivak and his associates (1958), made the following statement (in translation): "It is not proven that an effective immunization necessarily accompanies this production of serum antibodies, hemagglutinins, agglutinins." Nothing was said about MPI. The views expressed above have been repeatedly challenged. In 1944, Jawetz and Meyer had already proved that the whole blood of plague-immune animals can destroy a much greater number of plague bacilli than the blood of normal animals. This higher activity is primarily inherent in the plasma, not in the cells. In 1953, Meyer observed that a single inoculation of 2,500 viable E.V. bacilli stimulates antibody production rather irregularly in heterogeneous small groups of guinea pigs, although the immunity determining life or death proved to be quite strong. In order to reduce the MPI to below 10 or even to below 5, vaccinations with at least 2-4 million plague organisms were required; this dosage conferred 100 percent protection, with no local reactions at the site of infection. Even more impressive are the convincing records collected during the past years from experiments on highly susceptible nonhuman primates -- langurs and _Cercopitheci_ -- and the multimammate mouse (_Praomys natalensis_). A definite correlation between high levels of serum antibodies and the acquired protection against plague infection in animals has been established. This factor may be accepted with considerable confidence in connection with the immunization of man against plague: any vaccine, whether live or killed, in large or small dosage, in varying frequency and spacing of inoculation and mode of administration (subcutaneous, cutaneous by syringe, single- or multiple-puncture), which fails to induce the appearance of serum antibodies has thus been proven nonprotective and even inadequate to sensitize human subjects to booster inoculations. The actual number of viable attenuated _P. pestis_ organisms introduced into the skin of man by the so-called scarification method practiced in the Soviet Union is unknown. No antibody studies or field trials have been reported. Soviet investigators are therefore unable to state whether their protective immunizations are truly effective. Their claim that live vaccines are superior to killed ones is based entirely upon their extensive experiments on guinea pigs and mice. Dissatisfaction with their results continues to prompt the search for a more "efficient" live attenuated _P. pestis_ isolate. What may be encountered with more invasive isolates will be discovered only when they are tested on highly susceptible nonhuman primates. Since merely 1-10th of the universally recommended dose of living E.V. (Paris) vaccine produces unpleasant local and systemic reactions when inoculated subcutaneously, and since smaller doses given cutaneously also elicit local reactions, it is reasonable to anticipate more severe reactions when the standard dose is administered. In fact, the trial experiment just described demonstrates once again that a live plague vaccine eminently protective to small laboratory animals is not a biological which confers immunity upon man with the least amount of resultant discomfort. Even if the vaccine proves to be "safe" in tests on guinea pigs and mice, it may not be innocuous to some humans, as indicated by experiments on non-human primates. The numbers of human subjects included in individual groups in this report are too small to justify valid conclusions, but it is worth noting that the local reactions which followed subcutaneous inoculation of E.V. Paris were of the same magnitude in both the previously vaccinated and previously nonvaccinated individuals. Basic vaccination with killed plague vaccines apparently fails to blunt the unpleasant side effects of live vaccines. The goal of elevating the protective efficacy of killed vaccine from 60 to 90 percent, and incidentally conferring resistance to aerosol-pneumonic plague by subcutaneously administered live plague vaccine, may be achieved -- but, unfortunately, only at the risk of undesirable vaccination reactions.

(C). E.V. 51f.

A subculture of the E.V. 76 vaccine strain, brought to the USA in 1951 after passage through guinea pigs previously inoculated with nontoxic doses of iron sulfate, fulfilled the safety and pathogenicity criteria stipulated by Girard, Korobkova, and other investigators. The PD50 was 3,600 for mice inoculated by the subcutaneous route, and 5-45 viable organisms for guinea pigs. This passage culture, preserved as a freeze-dried live vaccine known as E.V. 51f, unexpectedly proved highly pathogenic for African vervet monkeys (Cercopithecus aethiops) and langurs (Presbytis entellus), but not for Macaca mulatta. Three independent series of experimental clinical trials using dilutions of the vaccine on Cercopithecus sp. resulted in the following vaccination fatalities:

(1) Ethiopian species: Death/Total - 3/16 or 18 percent; 160 million organisms - 1 vaccination fatality; 16 million - 1; 1.6 million - 1; solid immunity correlated with MPI antibodies -- indices 0 to 10 in survivors challenged on 41st day.

(2) Kenya species: Death/Total - 10/20 or 50 percent; highest mortality with 218 million viable E.V. 51 organisms; complete immunity of survivors, challenged on 52nd day.

(3) South African Cercopithecus pygerythrus vaccinated in South Africa: Death/Total - 13/50 or 26 percent; highest (40 percent) mortality with 100 million and 100,000 organisms; 7/10

survivors resisted subcutaneous challenge on 166th day. MPI of 1 to 3 on the 28th day rose to 15 and 17 in the 5th month after vaccination.

(4) Langurs (Presbytis), vaccination fatalities: Two of 4 langurs succumbed on the 6th and 7th days to subcutaneous inoculation with a total dose of 707 million containing 130 million viable E.V. 51f organisms. In a second experiment 1 of 2 langurs each inoculated intradermally by single bifurcated needle-puncture charged with approximately 8,000, 80,000 or 800,000 living E.V. 51f, became fatally infected.

Since 170,000 viable bacilli caused no vaccination fatalities in Praomys natalensis, it was decided to conduct a clinical trial on human subjects using the single or multiple cutaneous puncture technique. Fifteen inmates, in the previous 2 months each cutaneously punctured with 28 million live E.V. Paris vaccine organisms, had reflected immunity status as follows: Inmate Si: HA,0; MPI, 13; Inmate Ap: HA, 1024; MPI, 7; Inmate Tr: HA, 64; MPI, 13; and Inmate G: HA, 0; MPI, 12. The 10 subjects not previously vaccinated had no histories of contact with plague and yielded no serologic evidence of such contact. Seven were inoculated by bifurcated needle-puncture charged with 530,000 bacilli (1 puncture), while 7 others were each given 4 punctures with 2,100,000 E.V. 51f (lyophilized since April 1968 -- 106 weeks, with a mouse PD_{50} of >208,000). Contrary to expectations, particularly in view of the nefarious history of the isolate in nonhuman primates, the inflammatory nodules -- their evolution, and their ultimate transformation to eschars and scars -- were slightly more pronounced than such reactions induced by the E.V. Paris vaccine in Phase I. The diameter of the nodules and blisters exceeded 10 mm (in 1 man, the nodule had attained a diameter of 25 mm on the 72nd hour). No sharply defined differences in extent and intensity of the reactions in previously vaccinated vs. previously nonvaccinated individuals were noted. However, a dose-reaction relationship was distinct: 2 million organisms caused slightly more marked inflammation, with more gradual fading. No discomfort or impairment of function was recorded during the course of repeated inspections. Certainly the reactions never reached the intensity observed when 28 million organisms were introduced by 4 punctures. The inmate "gossip-line" testified that in comparison, the E.V. 51f induced only mild local reactions. Axillary lymph nodes were never palpable. Systemic reactions were likewise absent, but the WBC were slightly elevated above the average baseline in 8/14 men at least 24-48 hours after inoculation, particularly in the group receiving the large dose of vaccine. A glance at the recorded antibody response indicates that the dosages, as in the case of E.V. Paris, were inadequate to stimulate significant titers in man.

The MPI (average 15) remained identical to those recorded before and 30 days after inoculation. No booster effect was noted. The impression is inescapable that in this experiment, E.V. 51f behaved in man like the parent isolate E.V. 76 in use in Paris and Madagascar (Report 20).

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LIST OF PERSONNEL RECEIVING CONTRACT SUPPORT

Richard V. Walker, Ph.D. (Ph.D. received in 1960) Asst. Res. Immunologist	July 1, 1960-June 30, 1965 (5 years)
David McNeill Lab. Technician	July 1, 1960-June 16, 1970
Lilia Evans Lab. Technician	April 1, 1966-May 31, 1970
Edward Higgins Lab. Technician	March 5, 1962-July 31, 1964
Dorothy Bennet Lab. Helper	June 26, 1961-October 8, 1965
Huel Washington Lab Technician	November 1, 1962-December 31, 1965.

APPENDIX A

Standard Procedures Recommended for Evaluation of Plague Vaccines

I. Manufacture of killed vaccines: See Minimum Requirements, Appendix B.

II. Preparation of freeze-dried living attenuated P. pestis: The strain selected after extensive experimental animal studies is grown on veal infusion agar at 28°C, suspended in 0.2 M phosphate containing 7.5% sucrose, 0.7% glutamate and 2.5% human serum, freeze-dried in vials, and maintained at -20°C. The content is reconstituted with physiologic saline and the number of live organisms determined either by plating of 10-fold dilutions of the suspension on the surface of blood agar plates or by preparing pour plates in hemolyzed blood agar. A vaccine is considered effective if it contains not less than 30% live attenuated P. pestis: 450 million in 1.5 billion according to the optical standard (human dose).

III. Immunogenic efficacy tests on animals:

A. Assay on mice:

1. Killed vaccines: Details in Appendix B.

2. Live plague vaccines: The "residual virulence" (LD_{50}) of live plague vaccines is determined by subcutaneous and intraperitoneal inoculation of the agar-grown or freeze-dried reconstituted vaccine. The number of living organisms in the vaccine dilutions is estimated by surface-plating of 0.2 ml on each of 5 blood agar plates from that dilution which will yield 30-100 colonies per plate (10^{-6} dilution of broth culture). Intravenous inoculations are made with 6-10-fold dilutions of 0.5 ml each into the tail veins of inbred white mice. Challenge of the survivors with the standard dose of virulent P. pestis as used in the mouse potency test determines the PD_{50} of a vaccine strain.

B. Assay on guinea pigs (pathogen-free inbred strain, preferably Hartley):

1. Killed vaccines: The basic mouse protection test may be adapted for use in a modified procedure on guinea pigs. Dilutions of the U.S.P. killed vaccine are emulsified in the syringe with equal amounts of incomplete Freund's oil adjuvant (1 part Arlacel A (mannide oleate) + 9 parts Bayol 55 (light petroleum oil)) and

inoculated intramuscularly. The efficacy of the vaccine is determined by subcutaneous challenge infection with LD_{50} 200,000 virulent P. pestis. The essential advantage of this test is the possibility of obtaining supplementary serologic data (HA, MPI, etc.) on animals in the series reserved for exsanguination at the time when others are subjected to challenge infection. With the U.S.P. vaccine, an ED_{50} rate can be obtained by grading doses at 1:100, 1:1,000 and 1:10,000. Groups of 6 guinea pigs are inoculated: 4 of each group are exsanguinated and 6 are challenged. Although the ratio of ED_{50} test vaccine to that of the standard vaccine is usually higher than in comparable mouse potency tests, variability in both tests is approximately the same. Preliminary tests by D. M. Eisler (personal communication, 1969) suggest that 20-fold increases in the dilutions ordinarily chosen for the mouse test, given in a schedule of 2 doses 1 week apart by the intraperitoneal route, followed by subcutaneous challenge 14 days after the second inoculation, may replace the vaccine in adjuvants.

2. Living attenuated plague vaccines: Assays of living plague vaccines must determine (a) the specific harmlessness, and (b) immunogenic efficacy of a strain.

(a) Tests for harmlessness: Ever since live plague vaccines were introduced as prophylactics, it has been stipulated that a suspension which, according to optical standards, contains 15-20 billion P. pestis injected subcutaneously into guinea pigs should cause merely temporary weight-loss, but no deaths. Since total bacterial estimates made with reconstituted freeze-dried vaccines are frequently inaccurate, the number of viable attenuated P. pestis in the inocula is determined by colony counts on blood agar surface-plates. Groups of 4 or more guinea pigs are inoculated subcutaneously as well as intraperitoneally. Use of the latter route may cause death between 8 to 48 hours due to the liberation of toxins from the mass of nonviable, together with the rapidly growing, living bacilli. Later deaths (up to 20 days) reveal autopsy lesions resembling those of septicemic plague combined with extensive pathological alterations of the parenchymatous organs. Guinea pigs surviving intraperitoneal inoculation may be tested for immunity; or preferably, some are sacrificed and examined for granuloma formation in the lymph nodes, liver and spleen, and their tissues cultured to determine persistence of the plague bacilli at the site of inoculation, status of lymph nodes, etc., with special reference to absence of attenuated plague bacilli in the heart blood and pulmonary tissues. Attenuated strains which produce metastatic plague-like lesions in the lung are either not sufficiently attenuated, or suitable clones have not been selected. These strains are neither "harmless" nor "inoffensive" and must be discarded.

Intracutaneous inoculation (0.1 ml) of guinea pigs with 10-fold dilutions of 100,000 to 10 viable organisms provides valuable information concerning the "residual virulence" of a vaccine strain. Daily clinical examination, pertinent measurements, even photographic recording of reaction at the inoculation site accompanied by progressive evolutionary enlargement and changes in consistency of the regional lymph nodes (sometimes biopsies), and occasional recording of the body temperature, all supply information concerning the invasiveness and multiplication of vaccine strains. Generally such tests are combined with the appraisal of the vaccine's immunogenicity.

(b) Tests for immunogenic efficacy: Three groups of 10-14 guinea pigs are inoculated by the subcutaneous, intraperitoneal or intracutaneous route with 10-fold dilutions of the approximate number of viable organisms determined by preceding pilot tests. Daily clinical examinations are made at least for the first 5 days and then at intervals on individual animals judged important by the early findings. By the 28th day after vaccination, a minimum of 4 guinea pigs from each group are exsanguinated and their sera tested for antibodies, while 6 of 10, together with 10 controls for each route of immunization, are challenged subcutaneously with 0.5 ml of a 10^{-2} broth culture (1,000,000 or LD_{50} 200,000) of virulent *P. pestis* (195/P). If the calculated PD_{50} is less than 1000, the strain is used in further experiments.

C. Tests on rabbits: The antibody-stimulating capacity of vaccines may be determined in rabbits. The effect of dosage, single or repeated inoculations, and reactions at the site of inoculation frequently reflect the type of level of antibodies which appear in the peripheral blood.

D. Assay on subhuman primates: The experimental appraisal of plague vaccines in these animals has assumed an increasing importance. The inherent, highly heterogeneous resistance of the macaque species (*Macaca mulatta* and *M. cynomolgus*) revealed in extensive experimental series, precluded any reproducible, clear-cut results regarding serologic reponse to vaccine inoculations and resistance to challenge by various routes (Taylor, 1933). The observations of Wyssokowicz and Zabolotny (1897) and members of the German Commission (1899) showed that clinical reactions and autopsy findings in Indian Hanuman langurs (*Presbytis* (*Semnopithecus*) *entellus*) following small-dose infections resembled those in man (Chen and Meyer, 1965). With the continuous invaluable assistance of the National Center for Primate Biology at Davis, California, the difficulties of importing, conditioning and developing the nutritional regimen required by this species have been eliminated.

Pilot studies and subsequent, more detailed studies, because of the high and relatively uniform susceptibility to plague in these langurs, have far exceeded their anticipated value. Young members of this species of subhuman primate are easy to handle; they may be regularly examined clinically, their blood frequently collected, and the regional lymph nodes examined by biopsy. Their response to vaccines, particularly the living attenuated type, is more informative than that of any other mammal. In practice, groups of adequately conditioned Presbytis are inoculated with the living agent in graded dosages chosen to test the adequacy for subsequent use in humans. Animals are checked daily for at least 10 days for the following factors: reactions at site of inoculation (erythema, induration, eschar formation, etc.); regional lymphadenopathy (nodal size, consistency, etc.); rectal temperature; periodic biopsy of lymph nodes; direct microscopy and culture; and periodic blood data (i.e., WBC, hematocrit, hemoglobin, and differential counts, usually with samples drawn 3, 5, and 8 days post-inoculation). Any animal revealing a rectal temperature above 40°C is bled for culture on both solid and enrichment media. Experience has demonstrated that an invasive living vaccine (even E.V. 76 Paris) will often induce a temporary septicemia in the langur as well as in the African green monkey. Up to the time of challenge, all animals are bled frequently for large amounts of serum with which the usual battery of tests (HA, MPI, etc.) is performed.

African vervet or green monkeys (Cercopithecus aethiops sp.), though less susceptible to plague than langurs, are more readily available, but require careful conditioning and treatment for latent infections (Shigella, Plasmodia, etc.). Males are aggressive and may cause serious bite wounds. Repeated handling, particularly under restraint, is not well tolerated; consequently clinical examination of sick animals is limited. Comparative studies on langurs, Cercopithecus, and Macaca to determine differences in response to vaccine inoculations are still in progress. For purposes of measuring the "residual virulence" of live vaccine strains, Cercopithecus may serve as a substitute for the langur. The number of experiments with killed vaccines is too small to justify an opinion regarding the usefulness of the African subhuman primate for immunological studies. It remains for future investigators to decide whether these or other species of monkeys to be studied for susceptibility to P. pestis will help to solve the problem of immunizing man against plague. A priori, it is believed that this will indeed be the case, since subhuman primates are much closer to man in evolution than the guinea pig or mouse. The pathophysiology, and especially the moot and unanswered question of antibody versus cell-mediated immunity, can be more readily studied and determined in a large subhuman primate than in a rodent.

The methods and procedures already tested and proven on rodents are used on subhuman primates; however, due to restricted availability, titrations of vaccines are limited to groups of 2 to 10 animals. Nevertheless, clinical observations, particularly when combined with frequent serologic examinations, have furnished important information concerning the safety and protective efficacy of live and dead vaccines. No vaccine preparation should be used on human subjects without prior detailed studies on at least one species of subhuman primate highly susceptible to P. pestis.

E. Clinical tests on human subjects: Every plague vaccine preparation proven safe and efficacious in animal experiments must be clinically tested on groups of human subjects in accordance with principles currently specified by Government agencies. Such tests require the constant supervision of qualified clinicians and a medical advisor familiar with plague. Medical facilities of certain State Departments of Correction are organized to provide this supervision, including regular clinical examinations, taking of temperatures, medical care if necessary, collection of specimens, blood testing, and preservation of specimens for referral to laboratories equipped to conduct special serologic examinations. Local and general systemic reactions are recorded twice daily for 5 days and then once daily for another 10 days. They are graded according to the following scheme: Local. None: No erythema, induration, or edema; slight tenderness to palpation, disappearing within 24 to 48 hours. Mild: Erythema (2 to 4 cm), but little or no induration; tenderness persisting longer than 48 hours, sometimes with erythema. Severe: marked local edema of entire arm, extending onto forearm; moderate marked erythema, with or without lymphangitis. General. None: No temperature elevation, chilly sensations, headache or other general symptoms. Mild: Headache, chilliness, general malaise or temperature elevation of more than 1°F not explained by a respiratory infection or other cause. Moderately Severe: Chills, temperature above 100.6°F, headache, general incapacitation.

The *in vitro* and *in vivo* serologic tests employed in the experimental evaluation of vaccines in rodents and subhuman primates are performed with samples of blood serum collected at 8, 16, and 28 days or longer after basic vaccination, and then before and 28 days after booster inoculation. The mouse protection indices will continue to be considered highly significant until other tests (lymphocytic blastogenesis, immediate and delayed cutaneous hypersensitivity to Fraction I) have been evaluated in pilot groups of 25 to 50 men. It is believed that MPI below 10 indirectly reflect relative immunity to plague infection if the indices are paralleled by high (>256 to 2048) hemagglutination titers. This deduction is warranted, since clinical experiments on langurs followed by severe challenge infection have established fairly close correlation between MPI and resistance to plague.

F. Techniques to measure plague vaccine efficacy indirectly (probably incompletely) by serologic tests:

1. Passive mouse protection test: The procedure developed by the Hooper Foundation laboratory (Meyer and Foster, 1948) is followed. At least 10 mice are given injections of 0.5 ml of the undiluted (or diluted 1:2 if the protection is strong) serum to be tested, into the tail vein, immediately followed by 100 LD_{50} of a highly virulent culture of *P. pestis* subcutaneously. The mice are observed for a period adequate to allow complete development of infection -- in this animal, about 14 days. Sera collected after recovery from plague infection confer considerable protection upon mice, as indicated by lower fatality rates and extension of the average survival time. The differences, first in the percentage of animals that die, and secondly in survival time, are not strikingly significant when considered separately, but taken together the two factors acquire a heightened importance. To facilitate comparison these two factors are expressed in a single figure, and the percentage of mice that die is divided by the average day of death. The quotient is then designated the "mouse protection index" (MPI).

2. Passive hemagglutination (HA test): This procedure, described by Cavanaugh *et al.* (1965) and Chen and Meyer (1966), employs sheep or human red blood cells treated with tannic acid and then sensitized with highly purified Fraction I *P. pestis* antigen. The method requires continuous checking and controls in order to exclude confusion with heterophile antibodies. This highly sensitive test merely measures auxiliary antibodies but not the degree of immunity conferred by a prophylactic agent. Likewise, as a simple *in vitro* test, the HA reflects the ebb and flow of antibodies and the correspondingly altered refractoriness. Laboratory workers exposed to the risk of infection are routinely tested for HA antibodies; whenever levels are low, reinoculations are indicated.

3. Complement fixation test: Originally developed by Chen, Quan and Meyer (1952), the CF test is now used in the micro CF technique (Chen and Meyer, 1966). Less sensitive than that of the HA test, the reaction is invariably demonstrated in relatively high titers (1:8 to 1:512) after recovery (late convalescence) from plague (McCrumb *et al.* 1955). When CF titers are demonstrated late, following vaccinations with live vaccines, subhuman primates proved solidly resistant or recovered from clinical disease. Killed vaccines rarely yield CF antibodies in titers above 1:8.

4. Agglutinins: Formalin-killed, repeatedly washed suspensions of virulent *P. pestis* strains forming 15-18% Fraction I are used in the customary macroscopic test, which is primarily chosen to check sera with questionable HA titers.

5. Opsonocytophagic and complement fixation tests with murine toxin may not yield reproducible reactions and hence are not routinely used.

APPENDIX B

Killed Plague – U.S.P. Vaccine: Specifications and Minimum Requirements

(1) Definition: The chemically killed plague vaccine (low in A and B substances) is a bacterial suspension prepared from highly virulent P. pestis 195/P isolated in 1948 from a case of septicemic plague during the Poona epidemic. The stock culture is kept on blood agar in the cold in screw-top tubes, with occasional passage through guinea pigs in order to maintain the virulence at an LD_{50} of 3-15 organisms for mice. Cultures freeze-dried at high vacuum are satisfactory, but there is some hazard involved in handling them.

(2) Preparation of culture medium (E medium): Ingredients required to prepare 1 liter of the final product: Beef Hearts, lean, ground, 454 gm; distilled water, 1000 ml; phytone, BBL, 5.0 gm; casamino acid, technical, 5.0 gm; sodium hydroxide solution, 1 normal, 5.0 ml; sodium chloride, RG (ACS), 3.0 gm; sodium sulfite, 10% solution, 2.5 ml; 1-cysteine hydrochloride monohydrate, C.P., 0.1 gm; yeast extract, Difco, 3.0 gm; agar - agar, Moroccan powder #600, 20 gm (agar is only included when plague vaccine (E medium) agar is to be made. The formula without agar is used to make plague vaccine (E medium) broth). One normal solution of NaOH (32 - 8950) or HCL (32 - 2600) is used to adjust the pH when required.

Filter the broth through a Seitz-type press equipped with Scott F100 or Cuno D-00 pads and LFHB paper. Dispense in 1" x 6" test-tubes (20 ml), 32-oz bottles (700 ml), 1 liter. Sterility test bottles (800 ml). Agar in Roux bottles (150 ml). Sterilize in steam for 25 minutes at 115-122°C.

(3) Preparation of basic concentrate (basic lot): Transplant a loopful of P. pestis culture, strain 195/P, on blood agar slants into 20 ml E medium broth. Incubate for 24 hours at 37°C. After 24 hours, inoculate 2 32-oz Rx bottles containing 600 ml of sterile E medium broth with a suspension of the culture, using 1 tube (20 ml) per bottle. Incubate for 24 hours at 37°C. After 24 hours, check each bottle for purity and reject any contaminated bottles.

After examination for contamination, inoculate each of 288 Roux bottles, containing 150 ml of E medium agar, with 1.5 ml of suspension taken from one of the Rx bottles containing the P. pestis suspension by means of an automatic syringe. After inoculation of the Roux bottles with the suspension, aseptically add 25-35 sterile glass beads (contained in a sterile tube) to each Roux bottle. Inspect each Roux bottle for cracks and rock each bottle to distribute the inoculum. Label each truck loaded with inoculated Roux bottles and spray truck with Roccal Solution (1:300) before it is moved to incubator. Incubate 72 hours at 37°C (bottles horizontal).

After 72 hours, aseptically add 25 ml of NSS to each Roux bottle. Briefly rock Roux bottles to roll beads over agar. Allow Roux bottles to soak horizontally with additional rocking and soaking to remove all growth from the agar. Inspect suspension in each bottle for contamination using gram stain and microscope. Discard any contaminated bottles. Using a sterile vacuum system, harvest the suspension from each of the Roux bottles not contaminated into a tared, sterile 9-liter bottle. Determine volumes by weight and add 37% formaldehyde to obtain a 0.65% formalin concentration in the pooled harvest, i.e.,

$$\text{Vol. in cc of Conc.} \times .0065 = \text{Vol. in cc of 37\% HCHO.}$$

Allow pool to stand at room temperature for 5 days (killing period) with intermittent shaking. After 5 days, the concentrate is tested as follows (Store at 2-5°C until off test):

From one of the 24-hour, 32-oz Rx bottles inoculated with the P. pestis strain 195/P, make a dilution of 0.1×10^{-6} (a level which will yield less than 100 organisms per inoculation). Inoculate each of 5 blood agar plates with 0.2 ml of this diluted culture and spread with a spreader. Incubate at 28° ±2°C and examine after 24 hours for any evidence of contamination (growth other than P. pestis). After 48 hours of incubation, obtain a colony count per plate. Calculate the approximate quantity of P. pestis organisms in the dilute inoculum portion used to seed each bottle of medium, i.e.,

$$\text{Organisms each inoculation} = \frac{\text{Total Colonies on 5 Plates} \times \text{Vol. of Dilute Inoculum/Bottle}}{\text{Total Vol. Plated (ml/5 Plates)}}$$

After a dilution which yields less than 100 organisms per inoculation has been determined, the Nutritional Adequacy Test (to prove the E medium lot will support growth of P. pestis) is performed as follows:

Inoculate 2 1-liter test bottles each containing 800 ml of E medium broth with, in our case, 0.1×10^{-6} dilution of P. pestis culture and incubate at 28° ±2°C until growth is visually evident. If growth is evident, Nutritional Adequacy Test passes and the lot may be used. If no growth is evident, the whole lot of E medium broth is discarded.

The Bacteriostasis Test (to prove viability test environment will allow multiplication of low number of P. pestis cells) is performed as follows:

Inoculate with diluted P. pestis culture, in our case 0.1 x 10^{-6} 4 1-liter test bottles each containing 800 ml of E medium broth to which has been added 2.5 ml (using one 10-cc pipette) of basic plague (i.e., killed plague concentration). Swirl test bottles to mix thoroughly. (Note: The Viability Test is run concurrently with the Bacteriostasis Test). Incubate the 4 test bottles at 28° ±2°C for 7 days. Examine the 4 test bottles visually for evidence of growth which should be present (i.e., from diluted culture) and take a gram stain from each bottle to show purity of culture. After 7 days, subculture each bottle, using 2 blood agar plates. Incubate plates at 28° ±2°C. After 24 hours, examine plates for contamination (i.e., other than P. pestis organisms) and after 48 hours, re-examine for P. pestis growth and no other. The basic plague passes the Bacteriostasis Test if (a) all 4 incubated bottles reveal the visual characteristics of P. pestis growth, (b) all incubated plates demonstrate growth of only P. pestis organisms, and (c) gram stain discloses only P. pestis organisms.

The Viability Test (to prove basic plague pool is free of viable organisms - able to produce), which is done concurrently with the Bacteriostasis Test, is performed as follows:

Inoculate each of 4 1-liter test bottles of E medium with 2.5 ml of basic plague (using one 10-ml pipette). Swirl to mix thoroughly. Incubate the 4 test bottles at 28° ±2°C for 14 days. After 14 days, examine the 4 test bottles visually for evidence of growth which should not be present. Gram-stain medium. No live organisms should be present. Subculture each bottle, using 2 blood agar plates. Incubate plates at 28° ±2°C. After 48 hours of incubation, examine for evidence of any growth. No growth should be present. The Viability Test passes if (a) all 4 incubated bottles reveal no evidence of contamination, (b) both incubated plates are free of any contamination, (c) gram stain demonstrates no growth of any organisms, and (d) Associated Bacteriostasis and Nutritional Adequacy Tests have passed satisfactorily.

The plague pool is now stored at 2-5°C in the non-production area, awaiting vaccine production.

(4) Estimation of bacterial count: The plague concentrate contains

approximately 10 x 10^9 organisms/ml, with prescribed limits of formalin concentration (0.03 to 0.05%). Formalin is diluted with sodium chloride U.S.P. containing 0.5% phenol. The following formula is used:

$$\frac{\text{Filling bulk}}{\text{Volume, in ml}} = \frac{(\text{organisms/ml in plague conc.} + \text{pl. conc. vol. (in 1 ml)})}{2 \times 10^{-9}}$$

The number of plague organisms is counted with a Coulter counter (model B) using a 30 μ orifice. The Coulter counting and Petroff-Hauser check counts are performed with plague vaccine diluted to approximately 1 x 10^6 organisms/ml in 0.03% EDTA and 0.9% NaCl and sonicated.

Uniformity may be checked by determining the nitrogen content, which is approximately 0.057-0.68 mg N for the E medium and 0.0735 mg/N for the old type vaccine. Petroff-Hauser count of old vaccine yields 0.0495 N.

(5) *Preparation of diluted vaccine*: Just prior to dilution, the concentrate is thoroughly mixed in a container for 2 hours on the shaking machine at 60 cycles/minute. Bring the diluted bulk to a final phenol concentration of 0.5% by adding the amount required (5.5 ml of 90% phenol solution for each 1000 ml of concentrate used) to compensate for the lack of phenol in the concentrate. Collect samples in 50 (2), 20 (2), and 10 (2) ml amounts for testing. Store either in bulk or in suitable vials at 2-5°C in a cold-room.

(6) *Sterility*: Several vials of the finished product are tested using 1 ml per 40 ml of NIH (80) Thioglycollate and 1 ml per 50 ml Sabouraud media at 37°C.

(7) *Toxicity and safety tests* are performed on guinea pigs (preferably of the Hartley strain): Each of 3 guinea pigs (weighing 300-400 gm) is inoculated intraperitoneally with the human dose (generally 1.5 ml). A series of 5 white mice (Swiss-Webster strain) are likewise inoculated intraperitoneally with 0.5 ml of the vaccine. All animals are observed for 7 days and must survive without significant symptoms or weight loss.

(8) *Immunogenic requirements of the vaccine*: The ratio of the test vaccine to the control reference vaccine must be 0.5 in mouse protection value.

(a) *Assay on mice (Mouse Protection Test)*

1. *Dose of vaccine*. Each lot of the finished product is

tested on 100 mice -- 20 mice for each of 5 graded 3-fold dilutions. For example, when Swiss Webster mice are used, the dilutions are: 1:15, 1:45, 1:135, 1:405 and 1:1215. Dilutions inoculated intraperitoneally in 2 doses of 0.2 ml each, with a 7-day interval between injections, are chosen so that the 50% mortality point will fall about midway between the highest and the lowest dilutions. Because of genetic variations in the animals, the actual dilution range to be used must be determined in advance for the strain of mice selected for the test.

2. Challenge dose. On the 7th day after the second inoculation of vaccine, the mice are challenged subcutaneously with 0.2 ml of a 10^{-4} dilution, or 2000-4000 virulent *P. pestis* 195/P. The challenge vaccine strain to be employed in the potency test should be of such virulence that the challenge dose will kill 100% of the 10 susceptible mice inoculated subcutaneously in the abdomen and that 1:10 dilution of the challenge dose will kill 80% of the 10 mice. For the sake of consistent uniformity, the challenge dose is standardized: A loopful of culture from the slant is sown in 5 ml of a broth known to possess good promoting qualities and incubated for 18-24 hours at 37°C; 0.5 ml of this culture is inoculated into another 5 ml of the same broth and cultured identically. The culture is diluted out in 1% peptone water and 0.2 ml is given subcutaneously as the challenge dose, preferably in the abdominal wall. Experience has demonstrated that a 1:10,000 dilution of the culture will give a total count of 2000-4000 organisms per 0.2 ml, which is the recommended dose. The count should be checked by a surface plate count of 0.2 ml on 5% blood agar, using the 1:1,000,000 dilution. The challenge infection is 7 days after the last immunizing inoculation.

3. Duration of the test. The mice are observed for 14 days after challenge. The greatest number of deaths occur on the 6th, 7th and 8th day, with only 1 or 2 deaths as late as the 13th and 14th day. The mice are autopsied and smears made from the spleens. They are stained with Wayson stain and retained for reference.

4. Virulence controls. Every test includes the subcutaneous inoculation of 4 groups of 10 mice each for control of the virulence of the challenge infection. They are given dilutions of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} (2,000, 200, 20 and 2) plague bacilli so that the LD_{50} can be calculated.

5. Control reference vaccines. The control vaccine is a formalin-killed acetone-dried preparation lyophilized in the amount representing 1 ml of vaccine, sealed in vials and maintained in the

cold away from light. The content of a vial is reconstituted with 1 ml sterile water. The 3-fold dilutions are prepared from this suspension in saline, inoculated in the same manner, and the animals challenged as with the vaccine being tested.

6. Evaluation of results. At the end of 14 days, the 50% end-point (PD_{50}) of the control vaccine is calculated by the method of Reed and Muench and compared with the 50% end-point of the test vaccine (concerning limitations of test, consult F. E. Buckland and R. H. Treadwell, 1961: A comparison of plague vaccines by the mouse protection test, J. Hyg. 59:49-56).

7. Information recorded in protocol. The protocol for the antigenicity test should record (a) date of vaccinations, (b) weights of mice at beginning of test, (c) date of completion of test, (d) dose of vaccine given per mouse, (e) number of bacteria/dose, (f) number of mice vaccinated with each dilution of vaccine for both test and control vaccines, and number of survivors in each group at the end of the 14 days, (g) number of nonvaccinated control mice challenged and corresponding number of deaths (for LD_{50} calculation), (h) 50% end-points of tests with control vaccine, with the ratio of reference vaccine to test vaccine, and (k) signature of responsible official.

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13. ABSTRACT

Experiments on highly susceptible nonhuman primates, clinical trials on human subjects and the recent experiences in Vietnam remove the stigma that killed plague vaccines are nonprotective against plague. The USP vaccine developed under the auspices of the Commission on Immunization and standardized according to a recognized assay method in the dosage of 1 ml of 2 billion formalin-killed aqueous suspension highly virulent P. pestis administered subcutaneously or by jet injection, followed by several intramuscular booster inoculations in smaller doses of 0.2-0.5 ml (400 million to 1 billion organisms), is a safe vaccine relatively free from side effects. Its protective efficacy is measured by the passive hemagglutination and mouse protection tests. The experimental use of highly susceptible nonhuman primates disclosed that the classical attenuated P. pestis E.V. 76 strain is invasive and that it multiplies; thereby producing tissue injuries. In human subjects, dosages of approximately 1-10th that recommended both in France and the Soviet Union result in measurable unpleasant clinical reactions; thus sharing with many other live bacterial vaccines properties which may entail risks for some individuals. It does stimulate hemagglutinins in man but no significant protective antibodies following a single inoculation of 52 million viable cells. In this dosage, it may act as a booster in human subjects previously inoculated with killed vaccine. Thus, as with killed vaccine, at least 2 inoculations may be required to ensure adequate protection. Clinical pilot trials on man with relatively small doses of live vaccines administered intracutaneously by puncture failed to stimulate protective antibodies, even in previously vaccinated individuals. Basic vaccination with killed vaccines fails to blunt the definitely objectionable persistent local reactions. Until the safety and "innocuousness" of live P. pestis E.V. has been conclusively proven clinical trials should be suspended.

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